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Listeria monocytogenes: Growth and Control in Vacuum-Packaged Ground Beef

by

Rupinder Panayach

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***Listeria monocytogenes*: Growth and Control in Vacuum-Packaged Ground Beef** submitted by Rupinder Panayach in partial fulfillment of the requirements for the degree of Masters in Science in Food Science and Technology.

Michael E. Stiles

Dr. M. E. Stiles
(Supervisor)

Lynn McMullen

Dr. L. M. McMullen

Fred Greer

Dr. G. G. Greer

Sept. 30/98

Dr. G. Finch

ABSTRACT

Growth of three strains of *Listeria monocytogenes* in vacuum-packaged ground beef was investigated using a central composite design with storage temperature (0 to 10°C) and fat content (11 to 39%) as two variables. *L. monocytogenes* failed to grow in meat obtained from a research abattoir because the natural microflora was dominated by bacteriocinogenic lactic acid bacteria. Growth of *L. monocytogenes* occurred in meat obtained from a commercial source and both temperature of storage and fat content were significant variables affecting their growth. Random amplification of polymorphic DNA showed that if growth of *L. monocytogenes* occurred in vacuum-packaged ground beef, all three strains grew. *Leuconostoc gelidum* UAL187 (bac$^+$), *L. gelidum* UAL187-13 (bac$^-$), or *Leuconostoc* sp. UAL280 (bac$^+$) controlled growth of *L. monocytogenes* in co-inoculated vacuum-packaged ground beef compared with control samples. The results of the present study have shown that *L. monocytogenes* has the ability to grow at refrigeration temperatures in vacuum-packaged ground beef and its growth can be prevented with known lactic acid bacteria as biopreservatives.

DEDICATION

To Mom, Dad, and brother Kanwaljit

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I would like to express my sincere gratitude to my supervisor Dr. Michael E. Stiles for giving me a chance to study in his lab and for his guidance, support, and time during my graduate studies.

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1. General Introduction

Vacuum and modified atmosphere packaging has been used for many years to extend the storage life of raw meats. Under these conditions lactic acid bacteria (LAB) dominate the meat microflora. LAB have traditionally been used to preserve foods, for example cheese and other cultured dairy products, fermented meats and vegetables. In recent years, consumer trends have shown a preference for foods that are minimally processed and contain fewer chemical preservatives. LAB have the potential for use as biopreservatives in various foods. Bacteriocin-producing LAB or their bacteriocins have been looked upon as an alternative to "chemical" preservatives so that foods can be kept in their "natural" form while extending their storage life and enhancing the protection against foodborne pathogens.

Meat and meat products are susceptible to growth of spoilage and foodborne pathogenic bacteria. The microflora that develops on meats depends on the contamination from abattoir, equipment, workers, animal skin/hide, time and other conditions of storage. It is, therefore, important to control growth of spoilage and pathogenic bacteria on meats during storage. Storage life and the growth of pathogenic bacteria on meat products depends on storage temperature, initial microbial load, pH and gaseous environment of the packages of meat. Refrigeration temperatures select for growth of psychrotrophic bacteria. Temperatures between 0 and 10°C are not uncommon during storage, transport, processing, and retail display (Greer *et al.*, 1994) and thus time taken for psychrotrophic bacteria to grow to numbers that lead to spoilage varies, depending on the storage temperature. Depending on the hygienic practices during slaughter, cutting, processing, and storage, the type and level of initial microbial load will vary. Therefore, to extend the storage life it is necessary to keep the initial microbial load as low as possible. The initial microflora consists of a wide range of microorganisms that may include spoilage and pathogenic bacteria. Spoilage bacteria of meat include: pseudomonads, enterobacteria,

lactobacilli, *Brochothrix thermosphacta*, and *Shewanella putrefaciens* (Gill and Molin, 1991). Pathogenic organisms associated with meat include: *Salmonella*, *Staphylococcus*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Clostridium perfringens*, and *Escherichia coli*. Depending on the gaseous environment of the meat packages, the types of microorganisms that grow will vary. During aerobic storage of chilled meats, gram-negative bacteria such as pseudomonas type bacteria dominate (Shelef, 1980; Dainty and Mackey, 1992) and cause spoilage of meats. On the other hand, anaerobic storage of meats inhibits growth of aerobic spoilage bacteria and allows LAB to prevail mainly due to the elevated levels of carbon dioxide in anaerobic and vacuum-packaging (Enfors *et al.*, 1979). The dominance of LAB in anaerobically stored meats results in the extension of storage life compared with spoilage during aerobic storage. Raw meats stored aerobically have storage life of only 4 to 5 days, while vacuum-packaging extends storage life of fresh ground meats at refrigeration temperatures to several weeks. The emergence of psychrotrophic pathogens such as *L. monocytogenes* and *Y. enterocolitica* has raised concerns about the safety of anaerobically stored meats because refrigerated storage may allow the time necessary for these pathogens to reach high levels before products are consumed.

These factors have resulted in attempts to isolate LAB that can give predictable storage life and can control growth of psychrotrophic pathogens and has thus resulted in research on biopreservation of meats. Biopreservation is defined as the use of natural microflora of foods or their metabolic products. It has two aspects: it should result in the extension of storage life and enhancement of safety (Stiles, 1996). Storage life of the vacuum-packaged fresh meats can be increased if known LAB with the ability to control growth of other spoilage organisms are used as "starter cultures". By doing so the storage life of such products can be increased and predicted. To be successful as a biopreservative, LAB should ensure that the growth of psychrotrophic pathogenic bacteria, such as *L. monocytogenes*, does not occur during the extended storage period. As a result the overall

objective of the present research was to investigate the safety of raw meat with an extended shelf-life.

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2. Literature Review

2.1. POTENTIAL OF LACTIC ACID BACTERIA AS BIOPRESERVATIVES

LAB are a group of gram-positive, non-sporulating, microaerophilic bacteria that have a number of characteristics that give them a competitive advantage over other bacteria in anaerobic environments. Some of these organisms can grow at refrigeration temperatures, and produce antibacterial substances, and they are tolerant of the antibacterial effects of carbon dioxide. Lactic acid is the main product of carbohydrate metabolism of LAB (Kandler, 1983). *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella* are the main genera of LAB. Traditionally, LAB have been used as starter cultures for the fermentation of foods because they impart, flavour and retard growth of spoilage and pathogenic bacteria. The preservative action of LAB can be attributed to the production of organic acids (lactic and acetic acids) which lower the pH of foods, and other inhibitory substances such as hydrogen peroxide, diacetyl, and bacteriocins. These substances are antagonistic to many pathogenic and spoilage microorganisms. Hydrogen peroxide is only produced under aerobic conditions and diacetyl is produced by oxidation. Therefore, production of acids and bacteriocins are of special importance for the inhibitory effects of LAB under anaerobic conditions. Bacteriocin-producing LAB have the potential for use as preservatives. A number of bacteriocins of LAB have been reported that are active against food spoilage and pathogenic bacteria, including: *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Staphylococcus aureus*. Bacteriocins are thought to provide a competitive advantage for a producing organism against others within the same ecological niche (Ahn and Stiles, 1990) and to improve quality, extend storage life, and inhibit foodborne pathogens of vacuum-packaged ground beef.

Lactic and acetic acids are the main organic acids produced by LAB. Production of these acids in meats results in lowering of pH that gives LAB a competitive advantage over

the growth of other bacteria. It is this reduction of pH that plays a major role in safety and sensory attributes of fermented sausages, but this property would be a problem in vacuum-packaged fresh meats where changes in sensory attributes are not desired. Gram-negative bacteria are also sensitive to organic acids produced by LAB (Ray and Sandine, 1992) and *Enterobacteriaceae* are inhibited by low pH, lactate, and anaerobic environment (Grau, 1981).

Bacteriocins are antimicrobial proteins produced by bacteria, including LAB. Tagg *et al.* (1976) described bacteriocins as a heterogeneous group of antibacterial peptides produced by gram-negative and gram-positive bacteria. Due to increasing consumer demand for "natural" foods, bacteriocins or bacteriocin-producing LAB have attracted much attention. Bacteriocin-producing LAB have been isolated from meat and meat products and these include: *Lactobacillus sake* producing sakacin A (Schillinger and Lücke, 1989), *Carnobacterium piscicola* producing carnobacteriocins (Ahn and Stiles, 1990), *Leuconostoc gelidum* producing leucocin A-UAL187 (Hastings and Stiles, 1991). The main focus of bacteriocinogenic LAB has been to control *L. monocytogenes* (Walker *et al.*, 1990; Buchanan and Klawitter, 1992; Degnan *et al.*, 1992; Foegeding *et al.*, 1992). The effectiveness of LAB in foods depends on the type of food and its storage conditions. For chill stored meats, bacteriocinogenic LAB should grow at such temperatures and produce bacteriocin during their early growth phase to control unwanted microorganisms, including foodborne pathogenic bacteria, and should not impart undesirable sensory changes to meats.

2.2. *Listeria monocytogenes* - A CONCERN FOR FOOD SAFETY

L. monocytogenes is a gram-positive, psychrotrophic, and facultatively anaerobic bacterium that is widely distributed in the environment. Listeriosis is the disease caused by *L. monocytogenes*. Clinical signs of the disease range from mild 'flu-like symptoms to septicaemia, meningitis, endocarditis, and can result in abortions or still births in pregnant

women. Infections are usually associated with immunocompromised people, including pregnant women, and cause meningitis or meningoencephalitis (Schuchat *et al.*, 1991). However, many patients who were normal, healthy people or had conditions that are not considered immunosuppressive, such as heart or renal disease and diabetes, have become ill with listeriosis in both foodborne epidemics and sporadic cases (Schlech *et al.*, 1983; Azadian *et al.*, 1989; Schuchat *et al.*, 1992). *L. monocytogenes* became the object of considerable public concern because of the high mortality rate associated with listeriosis. The United States Centers for Disease Control and Prevention (CDC) estimated that 1,850 cases of listeriosis occur in United States every year, resulting in 425 deaths (Schuchat *et al.*, 1992) and in Canada 56 cases of listeriosis were reported in 1993 and 46 in 1994 (Farber and Harwig, 1996).

2.2.1. *L. monocytogenes* - a Foodborne Pathogen

L. monocytogenes is recognized as an important foodborne pathogen after being implicated as the causative agent of both epidemic and sporadic outbreaks of foodborne illness. The first major foodborne outbreak of listeriosis occurred in Nova Scotia in 1981 (Schlech *et al.*, 1983) involving 41 cases with a 27% mortality rate. Coleslaw was implicated as the source of *L. monocytogenes* because epidemic strain 4b was isolated from coleslaw obtained from a patients refrigerator. Cabbage fertilized by manure from sheep with listeriosis and kept for long periods of cold storage was the source of the listeriae. Subsequently, *L. monocytogenes* was documented in several additional outbreaks and sporadic cases (Schlech *et al.*, 1983; Fleming *et al.*, 1985; Linnan *et al.*, 1988; Schwartz *et al.*, 1988; McLauchlin *et al.*, 1990). Other well-documented outbreaks of listeriosis have been associated with consumption of a number of foods, including: pasteurized milk, raw milk, cheese, and paté (Farber and Peterkin, 1991; Gahan and Collins, 1991). Although red meats have not been involved in outbreaks of listeriosis, a few sporadic cases of listeric illness have been epidemiologically linked to uncooked hot dogs and undercooked chicken

(Schwartz *et al.*, 1988), paté (Morris and Ribeiro, 1989), and contaminated turkey franks (Barnes *et al.*, 1989). All 13 serovars of *L. monocytogenes* may cause human listeriosis but most cases have been caused by serovars 1/2a, 1/2b, and 4b (Farber and Peterkin, 1991). These incidents have heightened the awareness of *L. monocytogenes* as a public health problem and have resulted in increased efforts to prevent further outbreaks.

2.2.2. Occurrence in Meats

Occurrence of *L. monocytogenes* on raw meats, prevalence both in slaughterhouses and in the processing environment have become a major concern for food industries and public health authorities in Europe and North America (Doyle, 1988; Genigeorgis *et al.*, 1989; Gobat and Jemmi, 1990; Johnson *et al.*, 1990). This makes it impossible for the total removal of the organism from fresh meats and increases the potential for postprocessing contamination. Red meats, both beef and pork, have been investigated for the occurrence of *L. monocytogenes*. A survey of various foods recorded contamination with *Listeria* spp. on 9 of 16 (56.3%) chicken legs, 38 of 44 (86.4%) ground meats, and 6 of 30 (20%) fermented sausages (Farber *et al.*, 1989b). Another study reported that *Listeria* spp. on fresh meat can range up to 68% and on ground meat products can range up to 92% (Johnson *et al.*, 1990). Up to 50% of samples of ready-to-eat meat products tested in a study in Europe and Canada were contaminated with *Listeria* spp. Grau and Vanderlinde (1992) detected listeriae on 93 of 175 samples of vacuum-packaged processed meats obtained from retail stores and 5% of the samples contained more than 1000 colony forming units (CFU) per gram. Several kinds of foods are known to carry this pathogen, and it is believed that foodborne transmission may account for a substantial portion of sporadic cases of listeriosis (Pinner *et al.*, 1992; Schuchat *et al.*, 1992). Meat and poultry may play an important role in the epidemiology of listeriosis because of the occurrence of the pathogen in up to 92% of retail samples of ground beef (Johnson *et al.*, 1990).

2.3. GROWTH OF *L. monocytogenes* AT REFRIGERATION TEMPERATURES IN MEATS

Widespread distribution in the environment and frequent isolation of *L. monocytogenes* from meat and poultry products raises concerns about its growth, especially in meat products stored for longer periods of time at refrigeration temperatures. The reason behind this concern is that it can grow at temperatures between -0.4 and 50°C (Juntilla *et al.*, 1988; Ryser and Marth, 1991). The organism can also survive freezing and dry conditions (Dickson, 1990), and it can tolerate very high salt concentrations (Seeliger and Jones, 1986). Initiation of growth of *L. monocytogenes* can occur from pH 5.0 to 5.7 at 4°C and from 4.3 to 5.2 at 30°C (Ahamad and Marth, 1989; Farber *et al.*, 1989a). Because meats have pH values higher than 5.0 and have the essential nutrients to support growth of *L. monocytogenes*, meats (including poultry) are expected to be a good environment for growth of *L. monocytogenes*. It is, therefore, important to evaluate how *L. monocytogenes* behaves under various storage conditions. Contradictory results about growth of *L. monocytogenes* in meats at refrigeration temperatures have been reported by different authors.

2.3.1. Absence of Growth of *L. monocytogenes* in Meats

There was no growth of *L. monocytogenes* strain Scott A in ground beef (hamburger), irradiated hamburger, sausage, or chicken salad when stored at 4°C for 7 days (Buchanan *et al.*, 1987). Johnson *et al.* (1988) reported survival but no growth of strains Scott A and V-7 of *L. monocytogenes* in ground beef (pH 5.6 to 5.9) stored in oxygen-permeable or vacuum-packages held at 4°C for 14 days. Growth of three strains of *L. monocytogenes* (Scott A, Brie-1, and ATCC 35152) was not observed in ground beef (pH 5.8) or liver (pH 5.6) stored at 4°C for 14 days and 25°C for 1 day (Shelef, 1989). Addition of water, glucose, and calcium to beef samples resulted in no effect on the behaviour of *L. monocytogenes*.

The reasons for the absence of growth have been suggested to be the deficiency of nutrients in meats (Johnson *et al.*, 1988) or the association of necessary ingredients with the water-soluble portion of meat because growth of *L. monocytogenes* was observed in meat extract obtained after blending and centrifugation of meat samples (Shelef, 1989). The possibility of the presence of inhibitory substances against growth of *L. monocytogenes* in raw meat has been suggested (Buchanan and Klawitter, 1991). Growth of *L. monocytogenes* in irradiation-sterilized or raw vacuum-packaged ground beef stored at 5°C for 800 h was not observed, but it grew in cooked meat stored under similar conditions and it was suggested that cooking of meat may inactivate an inhibitory substance.

2.3.2. Growth of *L. monocytogenes* on Meats

In contrast to the studies in which no growth of *L. monocytogenes* in meats was reported there are studies that reported growth of the organism at refrigeration temperatures (Gill and Reichel, 1989; Glass and Doyle, 1989; Grau and Vanderlinde, 1990; Kaya and Schmidt, 1991). Growth of *L. monocytogenes* in/on meats is influenced by a number of factors and these are as follows:

2.3.2.1. Strain of *L. monocytogenes*

Strain differences are one of the most important reasons for conflicting results about the behaviour of *L. monocytogenes* in meats. Different strains of *L. monocytogenes* react differently under the same conditions (Barbosa *et al.*, 1995). They reported that strain Scott A (serotype 4b) did not grow in ground beef of high pH stored at 4°C while three other strains, N-7143 (serotype 3a), Na-19 (serotype 3b) and Na-16 (serotype 1/2a), increased in numbers by about 2 log CFU/g in 28 days of storage. When the same strains were inoculated into ground beef of pH about 5.5, only two strains increased by 2 log CFU/g in 35 days of storage, strain Na-16 (serotype 1/2a) survived and Scott A (serotype

4b) decreased by 2 log during 56 days of storage. They prepared inocula at 4°C to simulate contamination conditions in the cold environments of meat plants. Difference in growth of different strains was reported to be more pronounced as temperatures approached the minimum for growth of *L. monocytogenes* (Walker *et al.*, 1990). Most of the research on the behaviour of *L. monocytogenes* in foods, including meats, is focused on strain Scott A (Dickson, 1990; Buchanan and Klawitter, 1991; Harrison *et al.*, 1991) which is a clinical isolate and may fail to grow at refrigeration temperatures. A pool of different strains has also been used in studies where strain Scott A was not used (Glass and Doyle, 1989; Kaya and Schmidt, 1991).

2.3.2.2. pH of Meats

Another factor that has been shown to influence growth is pH of the meat samples. *L. monocytogenes* was capable of growth on vacuum-packaged beef of pH more than 6.0 stored at 0, 2, 5, and 10°C while it failed to grow below 5°C when packaged under 100% carbon dioxide (Gill and Reichel, 1989). Growth of *L. monocytogenes* depended on pH and it grew well in meat products with pH values near or above 6.0 while it grew poorly in products with pH at or below 5.0 (Glass and Doyle, 1989). Kaya and Schmidt (1991) reported growth of *L. monocytogenes* (serovars 4b, 1.2a, 1/2b, and 1/2c) on vacuum-packaged beef at 2°C in meat of pH greater than 6.0 but not on meat of pH less than 5.8. Growth of *L. monocytogenes* occurred in ground beef with pH greater than 6.0 (Gouet *et al.*, 1978; Gill and Reichel, 1989; Grau and Vanderlinde, 1990; Kaya and Schmidt, 1991) while no growth occurred in ground beef when pH was less than 6.0 (Buchanan *et al.*, 1987; Johnson *et al.*, 1988; Buchanan and Klawitter, 1991; Kaya and Schmidt, 1991). These differences in growth may have been directly due to the effect of pH or indirectly due to the production of inhibitory substances by lactic acid bacteria (LAB) as vacuum-packaged raw meats with pH less than 6.0 favour growth of LAB (Buchanan and Klawitter, 1992).

2.3.2.3. Storage Temperature of Meat

Growth of *L. monocytogenes* occurred in ground beef (pH >6) when stored at 0°C or above after vacuum-packaging but not under carbon dioxide (Gill and Reichel, 1989). Effect of temperature on growth of *L. monocytogenes* was shown in a study by Buchanan and Klawitter (1991) where the lag phase decreased when the temperature of storage increased from 0 to 2, 5, or 10°C. Temperature influenced growth of *L. monocytogenes* with growth reported at 7°C but not at 2 or 4°C (Kaya and Schmidt, 1991). Grau and Vanderlinde, (1990) reported that the growth of strain Murray B of *L. monocytogenes* was more rapid at 5°C than at 0°C.

2.3.2.4. Natural Microflora of Meat

The influence of natural microflora on the growth of *L. monocytogenes* has been speculated (Buchanan and Klawitter, 1991; Kaya and Schmidt, 1991). Lactic acid bacteria became the dominating microflora in beef (pH >5.6) stored at 2, 4, and 7°C for 9 weeks and no growth of *L. monocytogenes* was observed. When beef of high pH (pH >6.0) was used, *L. monocytogenes* grew at 2, 4, and 10°C due to growth of *Brochothrix thermosphacta* instead of lactic acid bacteria (Kaya and Schmidt, 1991). The authors concluded that growth of *L. monocytogenes* depended on the interaction of temperature, pH, and competing microflora. Gouet *et al.* (1978) reported that *L. monocytogenes* counts remained constant in monoxenic meat held at 8°C and *L. monocytogenes* counts decreased when co-inoculated with *Lactobacillus plantarum* and increased 1 to 2 log in the presence of *Pseudomonas fluorescens*. The pH of the samples inoculated with *L. monocytogenes* and *L. plantarum* remained the same. *L. monocytogenes* growth stopped in vacuum-packaged ground beef when the natural microflora reached maximum numbers suggesting that natural microflora can interfere with growth of *L. monocytogenes* (Gill and Reichel, 1989).

2.3.2.5. Gaseous Environment

A four log increase in *L. monocytogenes* Scott A was observed in vacuum-packaged lean beef stored at 5°C for 21 days with a lag phase of at least 7 days (Dickson, 1990). Equivalent rates of growth were found on both vacuum-packaged and air-stored beef. However, Carpenter and Harrison (1989) found slower growth on vacuum-packaged chicken compared with aerobic film-wrapped samples. *L. monocytogenes* grew in ground chicken under modified atmosphere packaging when 5% oxygen was included and storage was at 4, 10 or 27°C, but exclusion of oxygen from the package resulted in no growth of *L. monocytogenes* at 4, 10 or 27°C (Wimpfheimer *et al.*, 1990). Johnson *et al.* (1988) reported that *L. monocytogenes* failed to grow in ground beef stored at 4°C in oxygen-permeable bags.

2.3.2.6. Type of Meat Tissue

Growth of *L. monocytogenes* strain Murray B was affected by type of tissue (fat and lean tissue of beef strip loins) stored under vacuum (Grau and Vanderlinde, 1990). *L. monocytogenes* grew to higher levels on fat than on lean meat at both 0 and 5°C there was a lag phase before *L. monocytogenes* started growing on lean tissue. *L. monocytogenes* grew on fat tissue without a lag at -0.3°C and at higher temperatures, while the organism grew on pork muscle tissue only at temperatures of 15.4°C or higher during aerobic storage (Gill *et al.*, 1997). In contrast Chung *et al.* (1989) reported that *L. monocytogenes* strain Scott A grew better in lean than in fat tissue incubated at 5°C for a week.

2.3.2.7. Temperature of Inoculum Growth

Minimum temperature for growth of *L. monocytogenes* in chicken broth (pH 6.4) increased from -0.4°C to 0.5°C when the temperature at which the inoculum was grown was increased from 4°C to 30°C (Walker *et al.*, 1990). Incubation of inocula at 30°C resulted in more intense "cold shock" when inoculated into meat and stored at cold

temperature. Cultures were probably more sensitive to reductions in temperature after incubation at optimal growth temperatures. Similarly, Grau and Vanderlinde (1990) reported growth of *L. monocytogenes* Scott A at refrigeration temperatures after the inoculum was grown at 10°C. They concluded that the growth of an inoculum at higher temperatures may have resulted in long lag phases when the organism was stored at refrigeration temperature on chilled meats. However, Barbosa *et al.* (1995) reported that culturing of inocula at 4°C resulted in growth of strains of *L. monocytogenes* isolated from meat but it had no effect on growth of strain Scott A that either failed to grow or decreased in counts depending on the pH of beef samples.

The potential for growth of *L. monocytogenes* in vacuum-packaged ground beef exists but it depends on a number of factors such as the strain of *L. monocytogenes*, pH of the lean, storage temperature, and the type of tissue.

2.4. INHIBITION OF GROWTH OF *L. monocytogenes* BY LACTIC ACID BACTERIA

L. monocytogenes is a particularly difficult organism to control in food (especially meat) processing establishments because it can grow at refrigeration temperatures. Temperatures of 4 to 10°C are not uncommon in food distribution systems and in retail environments. Storage temperatures in home refrigerators average close to 7°C (Juntilla *et al.*, 1988). All of these conditions and characteristics of the organism make *L. monocytogenes* a potential problem in extended shelf life products. Most of the control measures are aimed at minimizing the levels of *L. monocytogenes* in raw meats, reducing cross-contamination of ready-to-eat meat products, and preventing growth in meat products (Tompkin, 1990). Biopreservation could provide a hurdle for growth of *L. monocytogenes* in meats and is gaining importance because it is a "natural" means of controlling pathogenic and spoilage microorganisms. Bacteriocin-producing LAB have the potential to control *L. monocytogenes* and these have been tested to control *L. monocytogenes* in various meat products (Yousef *et al.*, 1991; Degnan and Luchansky,

1992; Degnan *et al.*, 1992; Degnan *et al.*, 1993). Bacteriocin-producing LAB can be used either as an inoculum in the meat or the bacteriocins produced by them can be added to meats.

Microgard™ is a commercially available product of skim milk fermented by *Propionibacterium freudenreichii* subsp. *shermanii* and then pasteurized. It is approved for use as a food preservative by the Food and Drug Administration and it is used in about 30% of cottage cheese products in the United States (Daeschel, 1989). The antimicrobial spectrum of Microgard™ includes gram-negative bacteria, gram-positive bacteria, yeasts and molds. The antimicrobial activity is caused by a heat-stable polypeptide that is protease-sensitive (Al-Zoreky *et al.*, 1991) and propionic acid. Microgard™ decreased growth of inoculated strains of *L. monocytogenes* on steam-sterilized fresh crab meat for 6 days at 4°C by 0.5 to 1 log but after 6 days the numbers increased to the original level of inoculation (Degnan *et al.*, 1994).

2.4.1. Bacteriocins and Control of *Listeria* in Meats

Potential of bacteriocins to control *Listeria* has been studied in a number of foods. Nisin is a bacteriocin produced by *L. lactis* subsp. *lactis*. It is important because of its relatively broad antibacterial spectrum that includes bacterial spores (Vandenbergh, 1993). Nisin was the first bacteriocin to be granted GRAS (generally recognized as safe) status in the USA. It is the only bacteriocin that is commercially available and it is used in at least 47 countries as a food additive and it has been successfully applied in dairy products (Vandenbergh, 1993). Nisin is known for its antibotulinum activity but attempts to replace nitrite with nisin were not completely successful (Rayman *et al.*, 1981). However, use of nisin in combination with nitrite has been shown to prevent outgrowth of *C. botulinum* spores in meat products (Rayman *et al.*, 1981). Nisin is of limited use in meats because it works best at acidic pH.

Bacteriocins produced by LAB associated with meats, such as *Pediococcus*, *Carnobacterium*, *Lactobacillus*, and *Leuconostoc*, have potential for use as biopreservatives. The bacteriocins that have been most extensively studied in meats are the pediocins produced by pediococci and PA-1/AcH is the mostly widely tested pediocin. Pediocins are reported to be more effective on meats than nisin. Pediocin PA-1/AcH has an inhibitory and bactericidal effect on *L. monocytogenes* associated with fresh meats and is more effective in reducing *L. monocytogenes* at 4°C than at 25°C in hot dog exudate. It reduced *L. monocytogenes* counts by up to 7 log CFU in ground beef, sausage, and other products (Yousef *et al.*, 1991; Degnan and Luchansky, 1992; Degnan *et al.*, 1992; Degnan *et al.*, 1993). Attachment of *L. monocytogenes* to fresh beef was reduced after treating with pediocin PA-1 and viability of attached *L. monocytogenes* was decreased by 1.0 to 2.5 log depending on the concentration of pediocin PA-1 used (Nielsen *et al.*, 1990). Pediocin powder applied to plastic packaging bags completely inhibited inoculated *L. monocytogenes* on poultry and meat samples stored at 4°C for 12 weeks of storage (Ming *et al.*, 1997).

The effectiveness of different bacteriocins in meats has been compared. The possibility of use of bacteriocins to control growth of *L. innocua* was investigated in fresh ground pork stored aerobically at 5°C (Murray and Richard, 1997). Nisin A was more effective in decontaminating inoculated pieces of pork than Pediocin AcH, but the effect of both bacteriocins was lost after 2 days of storage and *L. innocua* grew to the same levels as the control samples. Resistant strains were isolated from meats treated with Nisin A but not with Pediocin AcH (Murray and Richard, 1997). Concerns about use of bacteriocins in raw meats has been expressed because of the emergence of resistant populations and loss of bacteriocin activity by proteases of meats. Loss of nisin activity has been reported by other authors due to its interaction with proteins (Scott and Taylor, 1981) or lipids or phospholipids (Jung *et al.*, 1992). There are reports about retaining bacteriocin activity for longer periods of storage at refrigeration temperatures in fresh meats (Nielsen *et al.*, 1990)

and turkey sausage after storage for 60 days at 4°C (Luchansky *et al.*, 1992). Loss of activity of bacteriocins in meats is a problem that limits their use in meats and this problem of loss of activity in meats may be minimized by using bacteriocin-producing LAB.

2.4.2. Bacteriocinogenic Lactic Acid Bacteria and Control of *Listeria* in Meats

Use of bacteriocin-producing LAB as a biopreservative of meats has advantage over use of purified bacteriocins to control pathogenic bacteria because this will result in production of bacteriocin over a longer period of time. Bacteriocinogenic LAB have been studied to control growth of *L. monocytogenes* in meat products.

2.4.2.1. Application for Biocontrol

Lactobacilli, pediococci, and carnobacteria have been investigated as a means of controlling *L. monocytogenes* in meats. *Lactobacillus bavaricus* MN, a meat isolate, inhibited growth of three strains of *L. monocytogenes* in beef systems with the native microflora reduced by minimal heat treatment, vacuum-packaged and stored at 4 or 10°C (Winkowski *et al.*, 1993). *L. monocytogenes* was inhibited or killed depending on the initial inoculum of *L. bavaricus* MN. Native microflora did not inhibit growth of *L. monocytogenes*. Increased inhibition was observed by decreasing the storage temperature and addition of glucose-containing gravy. *Lactobacillus bavaricus* MN is a bacteriocin producer and the authors detected bacteriocin in the meat samples. Schillinger *et al.* (1991) reported that *Lactobacillus sake* Lb706 (sakacin A producer) provided up to 1 log increase in inhibition of *L. monocytogenes* in pasteurized minced meat in comparison to a nonbacteriocin-producing isogenic variant. This showed the importance of bacteriocin production by LAB to control *L. monocytogenes*. The same authors also reported that inhibition of *L. monocytogenes* in ground meat was less than in MRS broth and *L. monocytogenes* grew after initial inhibition of 7 days. This may have been caused by

adsorption of bacteriocins to meat surfaces or decrease in activity caused by proteases of meat and may represent problems for use of bacteriocinogenic LAB in raw meats.

Carnobacteria being associated with raw meats stored at refrigeration temperatures have been studied to control pathogenic bacteria by different authors. *Carnobacterium piscicola* LK5, isolated from a raw ground beef, was effective in controlling growth of *L. monocytogenes* where background microflora was reduced by either irradiation or thermal processing when meat was stored at 5°C than at 19°C (Buchanan and Klawitter, 1992). However, the organism was ineffective for controlling growth of *L. monocytogenes* in non-sterile beef. *Carnobacterium piscicola* DX, a bacteriocin-producer was studied as a biocontrol (Campos, 1997). *L. monocytogenes* grew uninhibited in control samples to 7 to 8 log CFU/g after 14 days at 4°C, 12 days at 8°C, and 4 days at 15°C and was inhibited by *Carnobacterium piscicola* DX, a bacteriocin-producer, by 3 log CFU/g. Growth of *L. monocytogenes* was also controlled by *Carnobacterium piscicola* 2818, a nonbacteriocin producer. However, inhibition of *L. monocytogenes* by bacteriocin producer was significantly higher than by nonbacteriocin-producer at 4 and 8°C in minimally processed vacuum-packaged chicken cubes with gravy (Campos, 1997). In the same study it was reported that higher temperature of storage at 15°C both strains of *C. piscicola* caused inhibition of 1 log CFU/g. Bacteriocin was detected in samples co-inoculated with *C. piscicola* DX and inhibition of *L. monocytogenes* was enhanced by bacteriocin production and storage at lower temperature (Campos, 1997).

The influence of pediococci on growth of *L. monocytogenes* has been reported by different authors and being mesophilic their growth at refrigeration temperatures will be limited but they can be effective during temperature abuse. Berry *et al.* (1991) has shown that addition of high levels of at least 7 log CFU of *P. acidilactici*/g to frankfurters can be effective in controlling growth of *L. monocytogenes*. The degree of inhibition depended on inoculum level of pediococci, gaseous atmosphere, and storage temperature. In another study *P. acidilactici* H (designated JBL1095) or pediocin AcH had no effect on the growth

of *L. monocytogenes*. Strain JBL1095 or pediocin AcH inhibited growth of *L. monocytogenes* in exudate stored at 25°C which, if left untreated, supported growth of *L. monocytogenes* (Yousef *et al.*, 1991). *P. acidilactici* (JBL1095) had no effect on the growth of *L. monocytogenes* at 4°C but it was effective when samples were subjected to temperature abuse. Pediocin-producing *P. acidilactici* (JBL1095) caused a decrease of 2.7 log of *L. monocytogenes* compared with a nonpediocin-producing (JBL42) strain when inoculated on to vacuum-packaged wieners that were temperature abused at 25°C for 8 days (Degnan *et al.*, 1992). No growth of listeriae or pediococci was observed at 4°C for 72 days so their effect will be limited during storage at refrigeration temperature while it will be effective during temperature abuse.

Lactobacilli and carnobacteria are organisms associated with refrigerated meats and they grow at refrigeration temperatures. These LAB have proved to be effective in controlling growth of *L. monocytogenes* at refrigeration temperatures in fresh meats where background microflora was reduced. Pediococci being mesophilic are not expected to grow in refrigerated meats and therefore their use to control *L. monocytogenes* is limited. Pediococci can be relied on as biocontrols only during temperature abuse or during fermentation of sausages. To control growth of *L. monocytogenes* as a biocontrol LAB should be able to grow and produce bacteriocin at the temperature of storage of meat.

2.4.2.2. Use of Lactic Acid Bacteria as Starter Cultures

Pediococci are organisms of interest particularly during fermentation of meats and they have been used as starter cultures for sausage fermentations. Reduction of *L. monocytogenes* by 2 log CFU/g with pediocin-producing starter culture was observed compared with 1 log CFU/g reduction with nonpediocin-producing culture (Berry *et al.*, 1990). Inhibition of *L. monocytogenes* was observed when pH of the sausage samples remained above 5.5 illustrating that bacteriocin production occurred independent of carbohydrate fermentation. At low inoculum levels, only the bacteriocinogenic derivative

resulted in extension of lag phase. Bacteriocinogenic *P. acidilactici* PAC 1.0 resulted in an additional decrease in *L. monocytogenes* counts by 1 log compared with nonpediocin-producing *P. acidilactici* during the fermentation and drying processes of dry sausages production (Foegeding *et al.*, 1992). They also observed that both bacteriocin and lactic acid were responsible for inhibition but not complete elimination of *L. monocytogenes*. Similar results describing a larger reduction of *L. monocytogenes* by pediocin-producing starter culture than by nonpediocin-producer culture were reported by Luchansky *et al.* (1992).

Pediocin-producing and nonpediocin-producing *P. acidilactici* were used as starter cultures for chicken summer sausage that was inoculated with a five-strain mixture of *L. monocytogenes* (Baccus-Taylor *et al.*, 1993). Pediocin-producing starter culture caused 2.6 log CFU/g decrease in *L. monocytogenes* counts as opposed to 1.2 log CFU/g decrease by nonpediocin-producing starter culture during fermentation. This difference is speculated to be caused by *in situ* production of pediocin. Listeriae were not detected by enrichment after cooking and storage at 4°C for 60 days or 30°C for 7 days. In another study, turkey summer sausage was fermented with *P. acidilactici* pediocin-producer (JBL1095) or non pediocin-producer (JBL1350) (Luchansky *et al.*, 1992). In spite of equal amounts of acids produced during the 12-h fermentation, *L. monocytogenes* inhibition was about 3.4 log with JBL1095 compared with only 0.9 log with JBL1350. Listeriae were not recovered after processing but pediocin was recovered from sausages for at least 60 days of storage at 4°C providing additional safety if post-processing contamination or insufficient processing occurs. Pediocin-producing cultures were shown to be ineffective in controlling *L. monocytogenes* in temperature-abused frankfurters at 4°C due to insufficient growth of pediococci at lower temperatures but when the samples were stored at 25°C there was 2.7 log reduction in number of *L. monocytogenes* (Berry *et al.*, 1991; Degnan *et al.*, 1992). These studies show that use of known bacteriocinogenic LAB has an advantage over the use of unknown indigenous cultures for fermentation because

they will extend storage life and improve safety of these meats. The starter cultures used for meat fermentations are pediococci and these are effective in fermented meats and not in fresh meats and/or meats stored at refrigeration temperatures.

2.5. USE OF *Leuconostoc gelidum* UAL187 AS A BIOPRESERVATIVE

Carnobacteria and leuconostocs are associated with chill stored meats and produce bacteriocin-like substances (Harding and Shaw, 1990; Hastings and Stiles, 1991; McMullen and Stiles, 1993) and, therefore, they are the microorganisms of choice for these meats. A number of these organisms produce bacteriocins against *L. monocytogenes* but their effectiveness in meats has not been studied. *Carnobacterium piscicola* LV17 produces bacteriocins active against other lactic acid bacteria and strains of *Enterococcus* and *Listeria* spp. but the organism's growth was unpredictable at 2°C in anaerobically packaged raw meats and it was not successful for controlling spoilage (Leisner *et al.*, 1995). In contrast *L. gelidum* UAL187 was suggested as a potential candidate for use as a biopreservative, because it does not cause spoilage of fresh beef (Leisner *et al.*, 1995). *Leuconostoc gelidum* UAL187, originally isolated from vacuum-packaged chill stored meats, produces a bacteriocin, leucocin A-UAL187, that is active against other LAB and three strains of *L. monocytogenes* (Hastings and Stiles, 1991). *L. gelidum* UAL187 is an important LAB that has biopreservative properties because it produces leucocin A-UAL187 early in the growth cycle and over a wide range of temperatures ranging from 1 to 25°C and pH values of 4.5 to >6.5. It has been reported that *L. gelidum* UAL187 inhibited growth of an added spoilage organism and inhibition was attributed to bacteriocin production (Leisner *et al.*, 1996). *L. gelidum* UAL187 has also been shown to control adventitious flora of vacuum-packaged ground beef after extended storage for 35 days at 4°C (Worobo, 1997). It also stabilized the red colour and flavour did not deteriorate compared with uninoculated control samples. *L. gelidum* UAL187 still needs to be studied for its effectiveness in controlling

growth of *L. monocytogenes* to meet both aspects of biopreservation, i.e., extension of storage life and enhancement of safety (Stiles, 1996).

2.6. OBJECTIVES OF THE RESEARCH

L. monocytogenes is one of the most important foodborne pathogens because of the high mortality rate associated with it and its ability to grow at refrigeration temperatures. Because the foodborne microorganism is widespread in nature and it is impossible to totally eliminate it from raw meats, prevention of growth appears to be the best way to prevent outbreaks. The use of bacteriocin-producing LAB has been shown to extend storage life of vacuum-packaged ground beef (Leisner et al., 1996; Worobo, 1997) and the extended periods for which the vacuum-packaged ground beef products are stored can lead to the risk of *L. monocytogenes* growing to high numbers before these products are consumed. LAB that produce bacteriocins active against *L. monocytogenes* could provide an additional hurdle to growth.

The objectives of the study were:

1. To determine the effect of temperature of storage and fat content of meat on growth of *L. monocytogenes* in raw vacuum-packaged ground beef (Chapter 4).
2. To evaluate the ability of bacteriocinogenic LAB to control growth of *L. monocytogenes* in vacuum-packaged ground beef and to improve or assure safety (Chapter 5).

Three strains of *L. monocytogenes* were proposed for use as a cocktail to inoculate meat samples. To be able to differentiate these strains, Random Amplification of Polymorphic DNA (RAPD) was evaluated as a differentiating method (Chapter 3).

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3. Selection and Differentiation of Strains of *Listeria monocytogenes* for Inoculation of Meat

3.1. INTRODUCTION

Listeria monocytogenes, a psychrotrophic foodborne pathogen, is a potential food safety concern and its growth in products with an extended storage life should be controlled. Reports about its growth at refrigeration temperatures in vacuum-packaged ground beef are conflicting. To understand growth behaviour of microorganisms, more than one strain should be studied so that the results represent behaviour of *L. monocytogenes* in general instead of only one strain. There are two possibilities for this, either inoculating each strain separately or inoculating a set of different strains as a "cocktail". The advantage of the latter approach is that it is time saving because results can be obtained from a single experiment. Strains of a cocktail should be compatible and to trace the response of all of the strains in inoculated meat samples a method of differentiation or typing is needed. Typing methods that are most commonly used to differentiate strains of *L. monocytogenes* are based on biochemical, genetic and serological characteristics or their bacteriophage susceptibility. Biochemical methods are useful up to species level, but they cannot be used to differentiate strains (Seeliger and Jones, 1986). Serotyping has limited application for differentiation because it is not sufficiently discriminating (Seeliger and Jones, 1986). Phage typing has been successfully applied to type *L. monocytogenes* in epidemiological studies (McLauchlin *et al.*, 1986) but it is not feasible for a study with a cocktail of *L. monocytogenes* because appropriate phages are not readily available.

Genotyping methods (based on variations in nucleic acids) have high discriminating power, they are rapid and easy to perform, reproducible, and they are able to differentiate similar but unrelated strains. The genotyping methods include: plasmid profile analysis, restriction fragment length polymorphism (RFLP), and methods based on the polymerase chain reaction (PCR). Plasmid profile analysis is a simple yet powerful tool for

differentiating bacterial strains, provided the strains contain plasmids. Restriction fragment length polymorphism (RFLP) is based on variations in the number and sizes of fragments of DNA generated after cutting the DNA with restriction enzymes. This method is highly discriminatory and easy to perform, but its disadvantages are that it requires a relatively pure and intact DNA and results are difficult to analyze because the restriction fragments are usually numerous and closely spaced.

Methods based on polymerase chain reaction (PCR) have a number of advantages over other genotyping methods because they are relatively fast and results are easy to interpret. Typing methods based on PCR include PCR-RFLP and random amplification of polymorphic DNA (RAPD). PCR-RFLP involves double primer amplification of a known sequence of the genome and then cutting the amplified products with restriction enzymes and comparing these products. PCR-RFLP assays have the disadvantage that they need previous nucleic acid sequence information as the basis for designing the primers. As a result, use of PCR-RFLP is generally limited to defined genes or repetitive elements for which nucleotide sequences are known. To overcome these difficulties the PCR-based RAPD method has been developed which does not require previous knowledge of nucleic acid sequence information. RAPD differentiates genomic polymorphism (genetic variations) among closely related bacterial isolates (Welsh and McClelland, 1990; Williams *et al.*, 1990).

The polymerase chain reaction is a molecular technique that results in the selective production (amplification) of large amounts of a chosen region of a DNA fragment of defined length and sequence from a small amount of target DNA, also called "template" (White *et al.*, 1989). PCR uses the ability of DNA polymerase to synthesize a complementary strand of template by extending an oligonucleotide primer (single stranded oligonucleotide that anneals to template and is the starting point of polymerization) that has annealed to the template. PCR requires a thermal cycler (programmable device that is used for heating) and a reaction mixture. The reaction mixture consists of target "template"

DNA, PCR buffer, $MgCl_2$, each of the four deoxynucleotides (dNTPs i.e., dATP, dTTP, dGTP, dCTP), oligonucleotide primers, and thermostable polymerase. For amplification of the target DNA to occur and to delimit the region of the "template" to be amplified, two short oligonucleotides (primers) must anneal to the "template" with their 3' ends facing each other (extension of DNA always occurs from their 3' to 5' end), one to each of the two strands of "template" that have separated during the denaturation step. If these two primers are positioned close enough the product generated by one primer after polymerization acts as a binding site for the second primer, and primer binding sites are doubled in number. PCR uses repeated cycles of primer-directed DNA synthesis that result in the accumulation of amplified target sequences. Each thermal cycle of PCR, has three steps: (1) a denaturation step in which the target DNA is incubated at high temperatures to separate the two strands of DNA so that primers can attach; (2) an annealing step in which the reaction mixture is cooled and allows the primers to anneal to their complementary target sequences; and (3) an extension step that is done at intermediate temperature allowing extension of primers to occur and resulting in the generation of a complementary DNA strand to the DNA template by DNA polymerase (White *et al.*, 1989).

Compared with standard PCR that requires two primers with known homology to template, RAPD relies on single primers of arbitrary sequences to randomly amplify short regions of the genome (template). Differences in the amplification patterns between closely related genomes indicate genetic polymorphism (variations) and thus can be utilized for genotyping. The highly variable but relatively simple patterns obtained after electrophoresis of the amplification products are used to differentiate strains. RAPD can not only be performed on purified DNA (Williams *et al.*, 1990) but also on untreated (Mazurier and Werners, 1992) or lysed cells without DNA extraction (Lawrence *et al.*, 1993). The amplification is performed at low stringency (annealing at 30 to 45°C and ≥2 mM $MgCl_2$), allowing the primer to anneal to several locations on the two strands of target DNA. A single primer that has no known homology to a genome can anneal to many

sequences for which the match is imperfect. When two such adequate but imperfect annealing sites occur within 200 to 4000 bp of each other, and in the correct orientation (5′ to 3′) on opposite DNA strands, the sequence between these sites can be amplified. The extent of sequence amplification will depend on the efficiency of priming at each pair of primer annealing sites and the efficiency of extension (Welsh and McClelland, 1990). Different RAPD patterns (polymorphism) between different species or within the same species can be attributed to three possible reasons (Williams *et al.*, 1990): (1) insertion or deletion between the primer target sites; (2) mismatches due to single base changes within the primer target sites; and (3) the deletion of one or both of the primer target sites.

Reproducibility of RAPD fingerprints has been shown to be a problem and it depends on a number of factors such as template DNA and primer concentration, concentration and source of *Taq* DNA polymerase, temperature of annealing, number of thermal cycles, $MgCl_2$-concentration, and type of PCR apparatus used (Bassam *et al.*, 1992; Welsh and McClelland, 1992; Meunier and Grimont, 1993). Presence of amplification products in control tubes, i.e., without template DNA, has also been reported (Williams *et al.*, 1990; Meunier and Grimont, 1993). This contamination does not affect the resulting pattern because these bands do not match the bands in RAPD patterns of the target DNA. Kerr *et al.* (1995) reported that unless RAPD is used in conjunction with another sufficiently discriminative technique, analysis using a single oligonucleotide primer may fail to distinguish between strains. They suggested that if three primers are used, RAPD could be used as a sole molecular typing system for comparing epidemiologically linked strains. Concentrations of 10^7, 10^6, and 10^5 cells per PCR reaction yielded the same profile with minor variations in the strength of bands showing that concentration of DNA does not affect RAPD patterns (Niederhauser *et al.*, 1994).

RAPD has been used successfully for differentiating strains of *Clostridium difficile*, *Lactobacillus*, *Listeria monocytogenes*, *Campylobacter jejuni*, and *Escherichia coli* (Mazurier and Werners, 1992; Mazurier *et al.*, 1992; McMillen and Muldrow, 1992; Cavé

et al., 1994; Kerr *et al.*, 1995; Cocconcelli *et al.*, 1997). The objective of the present study was to use RAPD to differentiate three compatible strains of *L. monocytogenes* for use as a cocktail in meat inoculation studies.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial Strains

The strains of *L. monocytogenes* used as a cocktail in this study were List4, HPB65 and 642. List4 was obtained from G. G. Greer (Agriculture and Agri-Food Canada, Lacombe Alberta), HPB65 and 642 were obtained from E. Daley (Health Protection Branch, Health Canada, Ottawa). List4 was originally isolated from pork processing equipment and HPB65 and 642 were originally isolated from meat. The strains were checked for bacteriocin production against one another by direct and deferred inhibition tests as described by Ahn and Stiles (1990).

3.2.2. RAPD Analysis

Strains of *L. monocytogenes* were subcultured in Trypticase Soy broth (BBL Microbiology Systems, Cockeysville, Maryland) supplemented with 0.6% yeast extract (TSB-YE; Difco Laboratories, Detroit, Mich.) and grown at 37°C. The method used was described by Lawrence *et al.* (1993) with some modifications. One ml of an 18 h culture of each strain was centrifuged (16,000 x *g*, 5 min), cells were washed in sterile saline (0.9% NaCl, wt./vol.), pelleted again by centrifugation (16,000 x *g*, 5 min), and the supernatant was discarded. Cells were resuspended in 100 µl of sterile Millipore water and lysed by heating in a boiling water bath for 10 min The lysate was cooled on ice and centrifuged (16,000 x *g*, 5 min). The supernatant was used as a source of DNA (template) in the amplification mixture. A ten nucleotide primer OPM-01 (5′-GTT GGT GGC T-3′) that was synthesized on an Applied Biosystems 391 PCR MATE DNA synthesizer was used.

For the amplification procedure, reaction mixture (50 µl) was prepared that contained 1.25U of DNA *Taq* polymerase (Perkin-Elmer Cetus), PCR buffer (10 mM Tris-HCl at pH 8.3 and 50 mM KCl) (Perkin-Elmer Cetus), 1.5 mM $MgCl_2$ (Perkin-Elmer Cetus), 200 µM each of the four deoxynucleotides (dNTPs i.e., dATP, dTTP, dGTP, dCTP), 1.38 µM of primer OPM-01, and 2.5 µl of the lysate (supernatant). The reaction mixture was overlayered with 50 µl of sterile mineral oil. The reaction mixture was cycled 44 times through the following temperature profile in a Hybaid OmniGene temperature cycler: 94°C for 1 min, 30°C for 2 min, and 72°C for 2 min, with a 1-min "ramp" time between the annealing and extension temperatures (this gave time for primer binding). This was followed by a final cycle at 94°C for 1 min, 30°C for 2 min, and 72°C for 10 min After completion of PCR amplification the samples containing amplification products were held at -20°C for gel electrophoresis.

To determine the RAPD pattern, 10 µl of the amplification products was mixed with 2 µl of the gel loading buffer and resolved by electrophoresis on 2% agarose gels (Agarose NA, Pharmacia Biotech, Ltd.) in Tris borate buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 100 V for 80 min Gels were stained with ethidium bromide (1.0 µg/ml) for 30 min and photographed under UV transillumination. λ DNA (Promega, Madison, Wisc.) was cut with restriction enzymes *Eco*RI and *Hin*dIII and was included as a molecular size standard. Samples that did not contain cell lysate were prepared as reference controls for each batch of samples. Patterns of amplified DNA obtained after electrophoresis were visually compared.

After the preliminary studies on individual strains of *L. monocytogenes*, RAPD was used as a method for differentiation of *L. monocytogenes* for colonies picked from PALCAM plates (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) supplemented with selective supplements (SR150E, Oxoid) after inoculating as a cocktail of three strains in vacuum-packaged ground beef (Chapter 4).

3.3. RESULTS AND DISCUSSION

None of the strains of *L. monocytogenes* was inhibitory to the others when checked for bacteriocin production by direct and deferred inhibition tests. This means that they were compatible for inoculation in meat as a cocktail, therefore, all three strains were used. The three strains were not typable by serotyping because they were all serotype 1a (determined by J. Farber, Bureau of Microbial Hazards, Health Canada, Ottawa). Plasmid profile analysis also could not be used as a differentiating tool in this study because plasmids were not found in strains List4 and HPB65.

The strains were analyzed by RAPD. Preliminary studies showed that the RAPD patterns obtained with whole cell lysates gave profiles with background smearing. This smearing was reduced by using the supernatant of the whole cell lysate as a source of DNA instead of the whole cell lysate. Reproducible RAPD patterns were obtained with the lysate supernatant. Although some faint bands were observed in the reference control lanes, these did not affect the RAPD patterns of the samples. The RAPD patterns for each of the test strains of *L. monocytogenes* were distinct (Figure 3.1) and they were arbitrarily designated as RAPD-types A, B, and C for *L. monocytogenes* strains List4 and HPB65 and 642, respectively. Some of the bands were common for the three strains but there were distinct major bands in RAPD profiles of List4 and HPB642 that helped in differentiation of three strains. List4 had a major band of less than 0.56 kbp and HPB642 had a major band of size about 0.95 kbp.

RAPD was performed on the colonies of *L. monocytogenes* isolated from meat samples of all treatments inoculated as a cocktail of three strains (Chapter 4). Figure 3.2 shows the RAPD profiles of ten colonies of *L. monocytogenes* isolated from samples of one treatment for the final day of storage. Out of ten colonies one colony was strain HPB642, two colonies were strain List4 and seven colonies were strain HPB65. RAPD profiles of colonies isolated from other samples were also determined (results not shown).

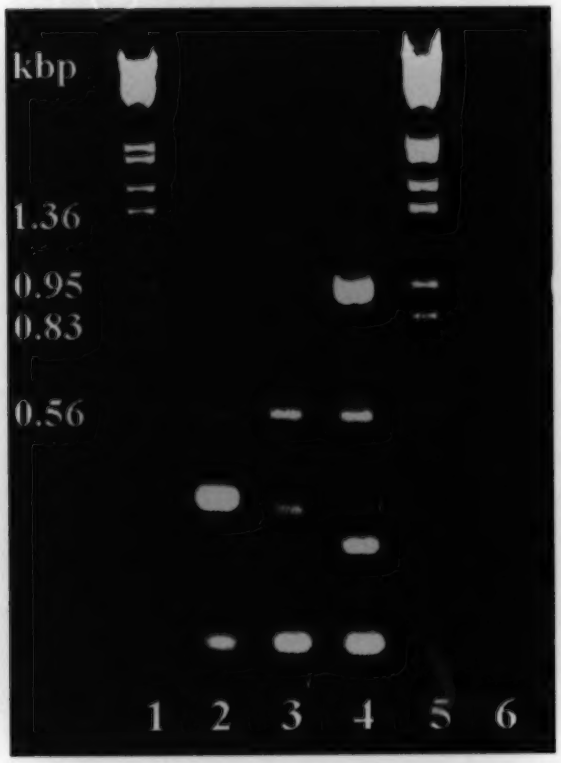

Figure 3.1. RAPD profile types obtained with primer OPM-01 for three strains of *L. monocytogenes*. Lanes 1 and 5 λ DNA molecular size marker (*Eco*RI and *Hin*dIII digest of λ DNA): 2, 3, and 4 RAPD types A, B, and C profiles for strains List4, HPB65 and 642, respectively: and 6 control.

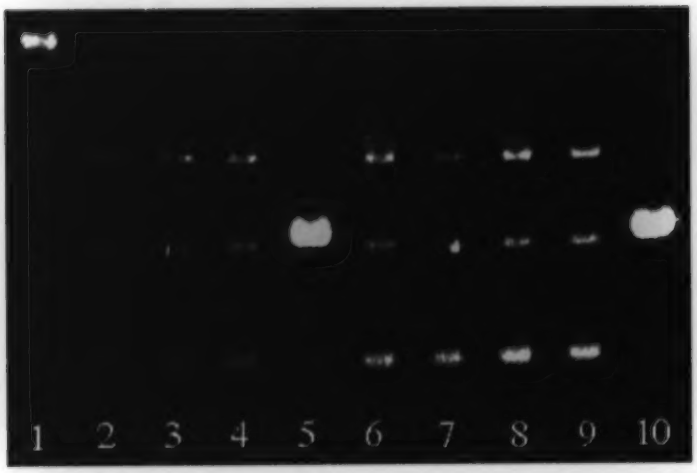

Figure 3.2. RAPD profile types obtained for ten colonies of *L. monocytogenes* isolated from meat samples that had been inoculated with a cocktail of three strains. Lane 1 RAPD profile for HPB642: lanes 2, 3, 4, 6, 7, 8, and 9 RAPD profile for HPB65: lanes 5 and 10 RAPD profile for List4.

Analysis of these RAPD profiles indicated that the three strains were present among the colonies of *L. monocytogenes* isolated from each treatment samples on the final day of storage and it lead to the conclusion that, whether growth of *L. monocytogenes* occurred in meat samples or not, all three strains of *L. monocytogenes* were detected at the end of the storage time. This indicated that for the treatments where growth of *L. monocytogenes* occurred all three strains of *L. monocytogenes* grew, but RAPD type B (HPB65) was generally detected more commonly than the other strains.

RAPD allowed the three selected strains that were otherwise indistinguishable by serotyping and plasmid analysis to be differentiated. RAPD has been used for typing of strains of *L. monocytogenes* and for the resolution of some epidemiological questions (Mazurier and Werners, 1992; Lawrence *et al.*, 1993) and as shown by the results of this study it can be used for research purposes where the tracing of the strains inoculated as a cocktail of *L. monocytogenes* was required. This confirmed that RAPD is a useful method for differentiating bacterial strains. It is a simple, rapid, relatively inexpensive technique and its results are easy to interpret.

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4. Growth of *Listeria monocytogenes* is Affected by Storage Temperature, Fat Content, and Native Microflora of the Vacuum-Packaged Ground Beef.

4.1. INTRODUCTION

Listeria monocytogenes is a potentially pathogenic microorganism that is widespread in the animal environment including slaughter houses (Genigeorgis *et al.*, 1989; Gobat and Jemmi, 1990). As a result it is a common contaminant of raw and processed meats. The incidence of *L. monocytogenes* in fresh meats ranges up to 68%, Pork is more commonly contaminated than beef or lamb. The prevalence of *L. monocytogenes* in ground meat and other products that require cooking before consumption ranges from 8 to 92% (Johnson *et al.*, 1990). Typical counts of *L. monocytogenes* in raw and processed meats range from ≤ 10 to 10^3 colony forming units (CFU) per gram (Breer and Schopfer, 1988; Buchanan *et al.*, 1989). *L. monocytogenes* is a psychrotrophic organism and, therefore, its ability to grow at refrigeration temperatures is of great concern for food products with extended refrigerated shelf-life. Although red meats have not been implicated in foodborne outbreaks of listeriosis, isolation of *L. monocytogenes* from raw meats makes these products a potential concern for food safety. Growth of *L. monocytogenes* on refrigerated vacuum-packaged meats could increase the risk of the organism surviving a mild cooking process and the hazard of cross-contamination of products that do not require cooking before consumption.

Several studies on the growth of *L. monocytogenes* on modified atmosphere packaged (including vacuum-packaged) meat and meat products has yielded conflicting results. No growth of *L. monocytogenes* was observed in vacuum-packaged ground beef stored at refrigeration temperatures (Buchanan *et al.*, 1987; Johnson *et al.*, 1988); however, Kaya and Schmidt (1991) reported that *L. monocytogenes* failed to grow in meat of pH < 5.8 but the strains grew in meat of pH > 6.0 at 2 and 4°C. Grau and Vanderlinde

(1990) reported that growth of *L. monocytogenes* on vacuum-packaged beef strip loins was dependent on the temperature of storage, the type of tissue (fatty tissue or lean), and pH of the lean meat. Similarly, Gill and Reichel (1989) reported that *L. monocytogenes* grew on beef strip loins at temperatures from 0 to 10°C. Barbosa *et al.* (1995) reported that growth of *L. monocytogenes* in vacuum-packaged top round ground beef at 4°C depended on the strains of the pathogen and the pH of the meat. Gouet *et al.* (1978) observed growth of *L. monocytogenes* on sterile meat only after the meat was spoiled by pseudomonads and pH had increased to > 6.2. In our laboratory, results of a study on growth of *L. monocytogenes* in ground beef confirmed that these bacteria have the potential to grow in vacuum-packaged ground beef (Bhatti, unpublished data). Growth of *L. monocytogenes* in vacuum-packaged ground beef could occur with pH of the lean and storage temperature having a major influence. Type of tissue has also been shown to affect growth of *L. monocytogenes*. There are ground beef products in the market with fat content up to 40%, but behaviour of *L. monocytogenes* in ground beef products with different fat contents at refrigeration temperatures has not been studied.

Response surface methodology (RSM) is a statistical technique for designing experiments, building models, evaluating the effects of factors, and searching for optimum conditions of factors for desirable responses (Myers, 1976; Montgomery, 1991). RSM is a useful method that overcomes the limitations of determining the effects of one variable at a time; that adequately describes the effects of the variables and their interactions; and allows variables of interest to be adjusted simultaneously in a single set of experiments (Box *et al.*, 1978). The equations derived from the data are called "approximating model equations" because they are approximations and not true representations of the response. The approximating model equations can be represented as response surfaces that can be used to describe how the variables affect the responses, to determine the interrelationships among the test variables, and to describe the combined effect of all test variables on the response. Although RSM is traditionally a product or process optimization technique, it

can be used to determine how the variables affect the responses at given levels of test variables and it has been used in studies of bacterial growth (Gibson *et al.*, 1988; Smith *et al.*, 1988; Buchanan and Phillips, 1990; Quintavalla and Parolari, 1993).

The objective of this study was to determine the effects and interaction of temperature of storage and fat content on growth of three strains of *L. monocytogenes* in vacuum-packaged ground beef of normal pH with a five level two factorial, central composite design.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial Cultures and Media

A cocktail of three strains (List4, HPB65 and642) of *L. monocytogenes* was used in the study. The strain List4, originally isolated from pork processing equipment, was obtained from G. G. Greer (Agriculture and Agri-Food Canada, Lacombe Alberta, Canada), and strains HPB65 and 642, originally isolated from meat, were obtained from E. Daley (Health Protection Branch, Health Canada, Ottawa). These strains were checked for bacteriocin production by direct and deferred inhibiton (Ahn and Stiles, 1990) and neither bacteriocin production nor antagonism between strains was found. All three strains were serotype 1a (determined by J. Farber, Bureau of Microbial Hazards, Health Canada, Ottawa). Stock cultures were frozen (-70°C) in trypticase soy broth (BBL Microbiology Systems, Cockeysville, Maryland) supplemented with 0.6% yeast extract (TSB-YE; Difco Laboratories, Detroit, Mich.) and containing 20% glycerol. Working cultures were maintained at 4°C in Cooked Meat Medium (CMM; BBL Microbiology Systems).

4.2.2. Ground Beef Inoculation and Sample Preparation

Fresh beef trim and fat were obtained from two sources, the research abattoir of the Lacombe Research Centre (Agriculture and Agri-Food Canada, Lacombe, Alberta) and a

commercial meat plant (Cargill Ltd., High River, Alberta). The meat trim and fat were vacuum-packaged and stored at -25°C until it was used in the experiment. Before grinding, the meat was thawed at 4°C for 18 to 24 h. Meat was coarsely ground (10 mm plate) with a meat grinder (Omas TS12, Model TS8, Stoney Creek, Ontario). Fat content of the trim and the fat was determined separately by total lipid extraction (Folch *et al.*, 1956) and calculated amounts of ground beef and fat were mixed to give the required fat content.

L. monocytogenes strains were grown separately at 25°C in TSB-YE and subcultured (0.1% inoculum) twice overnight at 25°C in 6 ml of TSB-YE before use in the experiment. One ml of an overnight culture of each strain was centrifuged at 16,000 × *g* for 4 min at 4°C, washed twice with 1 ml of sterile, 0.1% peptone water, resuspended and serially diluted with sterile, 0.1% peptone water to yield 10 ml of inoculum with approximately 6 log CFU/ml. The inoculum was distributed over the surface of the ground meat that was placed on a sheet of aluminium foil. Grinder parts that would come in contact with meat were autoclaved before grinding. The meat was ground twice through a 6.7 mm plate and 10 g samples were weighed and placed in bags made of high barrier film (Packall Packaging Inc., mylar/PVDC/polyethylene vinyl alcohol film; oxygen transmission rate 7.7 cc/m^2/24 h; moisture vapour transmission rate, 8.7 g/m^2/24 h). Each 10 g sample was vacuum-packaged (CVP Fresh Vac model A-300, CVP Systems Inc., Downers Grove, Illinois). Storage temperatures were selected based on central composite design and they were monitored with Mirinz-Delphi temperature data loggers (Tru-Test Distributions Ltd., Auckland, New Zealand).

4.2.3. Bacteriology

The microbial load of the ground beef samples was determined by testing duplicate samples of 10 g of meat blended with 90 ml of sterile, 0.1% peptone water for 2 min (Colworth Stomacher 400, Baxter Diagnostics Corp., Canlab Division, Edmonton,

Alberta). Appropriate dilutions were spread on the surface of prepoured PALCAM Agar Base (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) supplemented with selective supplement (SR150E, Oxoid) to determine presumptive *L. monocytogenes* counts and on Plate Count Agar (PCA, Difco) to determine total aerobic plate count. PALCAM plates were incubated aerobically at 37°C for 48 to 72 h and PCA plates were incubated at 25°C for 48 h. The mean log CFU for each sample was determined. Microflora of the ground beef will be distributed in the samples as opposed to on the surface if the samples were not made of ground product. So the pH of the meat sample homogenates prepared for plate counting was measured at the time of sampling using a Fisher Accumet pH meter. Peptone water (0.1%) does not have buffering capacity and it was shown that pH of the meat homogenate represents pH of the meat.

4.2.4. Differentiation of *L. monocytogenes* Colonies Isolated from Meat Samples

Random Amplification of Polymorphic DNA (RAPD) technique was used to differentiate the three strains of *L. monocytogenes*. RAPD was done according to Lawrence *et al.* (1993) except that the cell lysate was centrifuged and the supernatant was used in the reaction mixture instead of the lysate. RAPD was done on *L. monocytogenes* colonies isolated from PALCAM plates from the final samples from meat from the research abattoir for each treatment and on colonies isolated for selected treatments (11% fat at 5°C; 25% fat at 5°C; and 25% fat at 10°C) from samples on the last day of storage for meat from the commercial meat plant.

4.2.5. Antagonism of Background Microflora of Meat Samples

Colonies were isolated from PCA plates for the final day of storage from meat samples from both studies. These colonies were grown in APT broth and checked for bacteriocin production against three strains of *L. monocytogenes* and against one another by deferred antagonism (Ahn and Stiles, 1990).

4.2.6 Experimental Design

Two factorial Central Composite Design (CCD), a second order response surface methodology design (Gacula and Singh, 1984; Khuri and Cornell, 1996) was used to develop the levels of the independent variables for these studies. Data collected from these treatment combinations were used to develop a model. The model gives a mathematical relationship between independent variables and response variables (dependent variables). The independent variables were temperature (X_1) and fat content (X_2). The total number of design points for CCD with two variables is $N = 2^k + 2k + n_o$, where k is the number of variables and n_o is the centre point. All of the points (original values) of the design were given a coded value to facilitate the calculations. CCD design consists of three parts, factorial, centre, and axial points. Centre point, n_o, of the model is the middle of the experimental range of both variables and was assigned the variable code 0. The factorial points of the model represent high and low levels of each variable and were assigned the variable code $\pm$ 1.0. Axial points of the model represent the extreme limits of the variables under study and their inclusion allows for a better fit during regression modeling. They were assigned the variable code $\pm \alpha$, calculated by the equation $\alpha = F^{1/4}$ where F is the number of factorial points. When F is equal to 4, α is equal to 1.414 ($4^{1/4}$) (Gacula and Singh, 1984; Khuri and Cornell, 1996). The original values of the centre point for each independent variable were chosen and the original values of the factorial and axial points were calculated by choosing the intervals that give the maximum and minimum values of each variable. Correspondence between these coded and original (actual) values are given in Table 4.1. For a two factorial CCD there are nine design points. The centre point is duplicated to give a total of ten design points. Response measures considered for the studies were lag time, growth rate, time taken for 1 log increase in *L. monocytogenes* counts, maximum change in log CFU of *L. monocytogenes* per gram during the total period of storage (Max Δ log), and the change in log numbers of colony forming units of

Table 4.1. Independent variable levels (original values) corresponding to the coded variable levels of temperature and fat content of the treatments for a two factorial central composite design.

Independent variables	Variable levels at coded variables of:				
	-1.414[c]	-1[b]	0[a]	+1[b]	+1.414[c]
Temperature (°C)	0	1.5	5	8.5	10
Fat content (%)	11	15	25	35	39

[a] Centre point

[b] Factorial points

[c] Axial points

L. monocytogenes per gram after 10 days of storage (Δ log CFU). Each value was the mean of duplicate readings.

4.2.7. Data Analysis

The data were analyzed using response surface regression procedure of the Statistical Analysis System (SAS, 1990) to conduct RSM analysis and to fit the following second-order model equation for dependent variables:

$$\hat{Y} = b_0 + b_1x_1 + b_2x_2 + b_1 x_1^2 + b_2 x_2^2 + b_{12} x_1 x_2$$

Response surface plots were drawn to illustrate the main effects of the independent variables. Graph 3D procedure of the Statistical Analysis System (SAS, 1991) was the program used to draw the three dimensional graphs.

4.3. RESULTS

4.3.1. Difference of Results Between Two Experiments

Data for the growth of *L. monocytogenes* in meat from the research abattoir showed that a maximum of 1 log CFU/g increase occurred only with treatments 39% fat at 5°C; 35% fat at 8.5°C; and 25% fat at 10°C (Table 4.2). These results differed from those obtained in a separate study in our laboratory in which up to 4 log increase in *L. monocytogenes* was observed in vacuum-packaged ground beef at 10°C (Bhatti, unpublished data). This shows that growth of *L. monocytogenes* was inhibited in samples prepared with meat from the research abattoir. Deferred inhibition tests were performed to see if the background microflora contained bacteria that are inhibitory to growth of the three experimental strains of *L. monocytogenes*. The results of deferred antagonism for colonies isolated from PCA plates showed that the background microflora of these meat samples was dominated by bacteriocin-producing lactic acid bacteria. Of the sixteen colonies isolated, eleven were inhibitory to all three of the *L. monocytogenes* strains. These bacteria

Table 4.2. Differences of maximum change in log CFU/g of *L. monocytogenes* (Max Δ log) count in the two experiments using meat from the research abattoir and the commercial meat plant.

Temperature (°C)	Fat content (%)	Max Δ log*	
		Exp 1	Exp 2
10	25	1.03	4.12
8.5	35	1.36	4.12
8.5	15	0.61	3.27
5.0	39	1.39	3.37
5.0	25	-0.68	3.75
5.0	25	-0.72	3.56
5.0	11	0.12	2.74
1.5	35	-1.43	-0.08
1.5	15	-1.47	-0.87
0.0	25	-1.60	-0.46

Exp 1, meat from the research abattoir

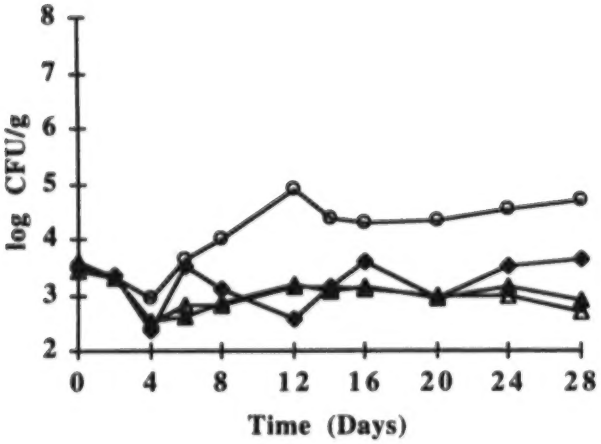
Exp 2, meat from the commercial meat plant

* Max Δ log was the maximum change in *L. monocytogenes* counts during 30 days of storage under the experimental conditions

were identified as *Leuconostoc* sp. by standardized biochemical tests. An identical experiment was done with meat from a commercial meat plant to determine whether the three strains of *L. monocytogenes* would grow in meat from a different source. In these meat samples, up to 4 log increase in *L. monocytogenes* counts was observed compared with only one log increase in *L. monocytogenes* counts in the previous study (Table 4.2). Bacteriocin-producing lactics were also isolated from meat samples from the commercial meat plant but they were not the dominating microflora of the meat.

Samples stored at 5°C were used for comparing the two studies because this was the lowest temperature at which growth of *L. monocytogenes* was observed. Growth of *L. monocytogenes* in meat samples from the research abattoir is shown in Figure 4.1A. In all samples stored at 5°C, pH dropped below 5.0 from an initial pH of 5.6 to 5.7 after 8 to 12 days of storage (data not shown), coinciding with the attainment of maximum population of the total bacteria (Figure 4.2A). The drop in pH was similar for samples stored at 5°C so it cannot be the reason for inhibition of growth. *L. monocytogenes* grew in samples prepared from commercial meat stored at 5°C with 11, 25, and 39% fat content with increases of log 2.74, 3.56, and 3.37, respectively (Figure 4.1B). Lag time of about 9 days was observed in samples with 11% fat content; while in samples with 25 and 39% fat, growth was detected within three days of storage at 5°C. The maximum population of *L. monocytogenes* reached was lower in samples with 11% fat compared with samples with 25 or 39% fat (Figure 4.1B). The pH of the samples dropped below 5.0 after 21 to 24 days of storage, coinciding with the attainment of maximum population (Figure 4.2B). The background microflora grew faster in samples from the research abattoir compared with the commercial meat samples, reaching maximum population in 12 to 14 days compared with 15 to 18 days in the commercial meat samples (Figure 4.2A and 4.2B). This comparison of growth at 5°C of the two experiments showed that there was a marked difference in growth of *L. monocytogenes* in meat from the two sources (Table 4.2). In both experiments, *L. monocytogenes* failed to grow at 0 or 1.5°C, despite the fat content of

Research Abattoir



Commercial Meat Plant

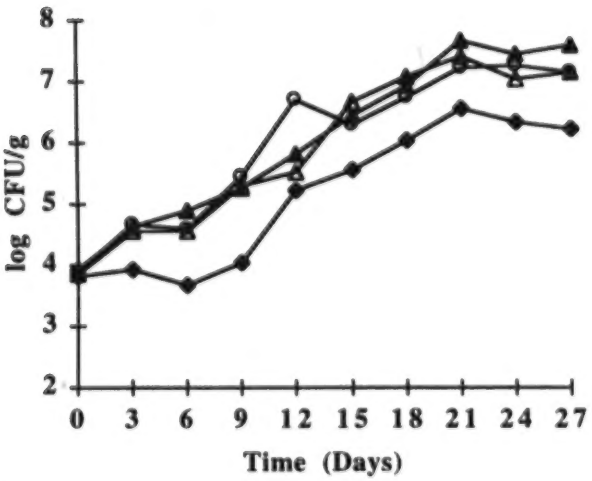


Figure 4.1. Changes in *L. monocytogenes* count in vacuum-packaged ground beef samples stored at 5°C. Meat samples with 11% fat content (◆), 25% fat content (replicate 1) (▲), 25% fat content (replicate 2) (Δ), and 39% fat content (O). Each data point is mean of duplicate samples.

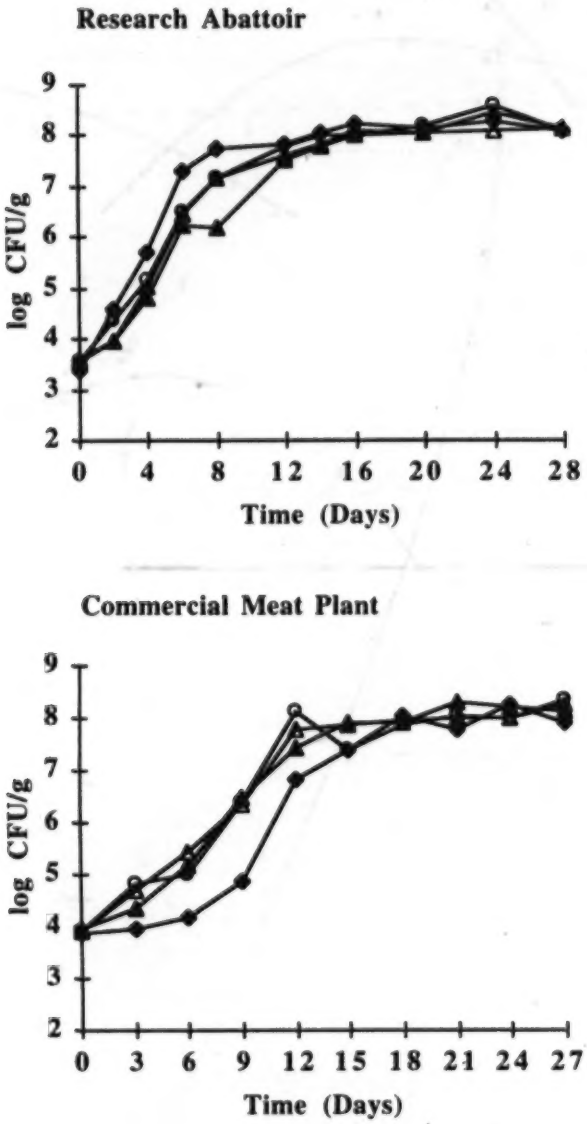


Figure 4.2. Changes in total count in vacuum-packaged ground beef samples stored at 5°C. Meat samples with 11% fat content (◆), 25% fat content (replicate 1) (▲), 25% fat content (replicate 2) (Δ), and 39% fat content (O). Each data point is mean of duplicate samples.

the meat. At storage temperatures of 5°C or above, dominance and faster growth of the background microflora by bacteriocin-producing organisms in meat samples from the research abattoir might have resulted in inhibition of growth of *L. monocytogenes*. Because growth of *L. monocytogenes* was inhibited in meat samples from the research abattoir, only data from commercial meat samples were used for statistical analysis.

4.3.2. Statistical Analysis and Response Surfaces

Lag time, growth rate, and time taken for 1 log increase in *L. monocytogenes* counts could not be used as response measures because of the lack of growth in some treatments. The change in log numbers of *L. monocytogenes* in 10 days of storage (Δ log CFU) and maximum change in log numbers of *L. monocytogenes* during the storage time (max Δ log) were used as responses for statistical analysis. Results for the dependent variables, Δ log CFU and max Δ log, are shown in Table 4.3. Δ log CFU ranged from -0.22 to 3.76 and max Δ log ranged from -0.08 to 4.12 showing that responses were affected by the variables. Table 4.4 summarizes the results of the analysis of variance for each of the dependent variables. The models for Δ log CFU and max Δ log (R^2 equal to 0.99 and 0.96 for Δ log CFU and max Δ log, respectively) were adequate, i.e., they were well adjusted to the experimental data and explained most of the variability in the responses with highly significant F values ($p \leq 0.01$). Moreover, the lack-of-fit test, which measures the fitness of the model obtained were not significant, indicating that the model equations accurately predicted the value of the dependent variables (responses) for any combination of the independent variables within the ranges studied. The main effects for each response were linear, i.e., first order. Second order or quadratic effects were significant at the 5% level for both of the responses accounting for the curvature in the response surfaces (Figures 4.3 and 4.4). Interaction between temperature and fat content was nonsignificant for both responses. Temperature was the single most important factor affecting Δ log CFU and max Δ log for *L. monocytogenes* and it was significant at the 1% level.

Table 4.3. Growth responses of *L. monocytogenes* in ground beef samples using meat from the commercial meat plant for ten treatment combinations of temperature and fat content.

Design Points	Independent variables		Response variables	
	Temperature (°C)	Fat content (%)	Δ log CFU	Max Δ log
1	10	25	3.76	4.12
2	8.5	35	3.10	4.12
3	8.5	15	2.45	3.27
4	5.0	39	1.56	3.37
5	5.0	25	1.35	3.75
6	5.0	25	1.46	3.56
7	5.0	11	0.23	2.74
8	1.5	35	-0.22	-0.08
9	1.5	15	-0.40	-0.41
10	0	25	-0.24	-0.46

Table 4.4. Analysis of variance for the responses of change of log CFU/g of *L. monocytogenes* in 10 days of storage (Δ log CFU) and maximum change in log CFU/g of *L. monocytogenes* during storage (Max Δ log) in samples using meat from a commercial meat plant.

Source	Degrees of freedom	Sum of squares		F-ratio	
		Δ log CFU	Max Δ log	Δ log CFU	Max Δ log
Regression					
Linear	2	18.39	26.28	180.7**	38.53**
Quadratic	2	0.78	5.42	7.63*	7.94*
Crossproduct	1	0.06	0.07	1.09^{NS}	0.12^{NS}
Total regression	5	19.22	31.76	75.53**	18.63**
Residual					
Lack of fit	3	0.20	1.35	10.89^{NS}	24.86^{NS}
Pure error	1	0.01	0.02	–	–
Total error	4	0.21	1.36	–	–
Factors					
Temperature	3	17.64	31.22	115.50**	30.52**
Fat content	3	1.32	1.69	8.67*	1.65^{NS}

** Significant at 1% level

* Significant at 5% level

^{NS} Nonsignificant

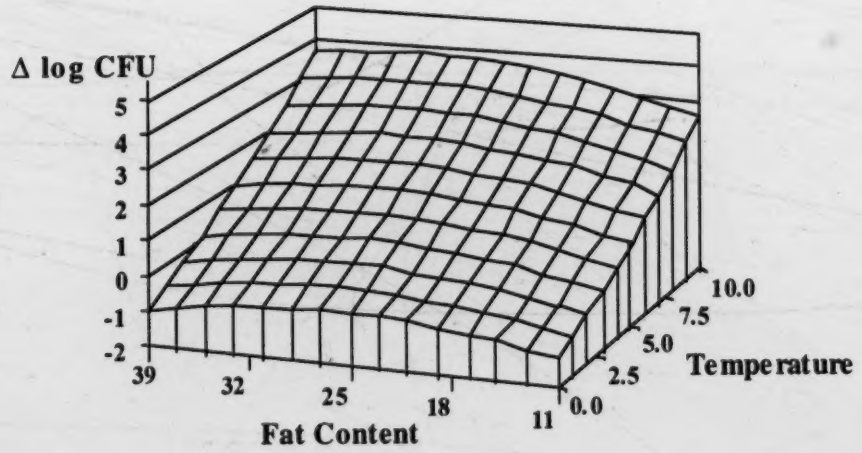


Figure 4.3. Effect of temperature of storage and fat content of the ground beef on the change of log CFU/g of *L. monocytogenes* in 10 days of storage (Δ log CFU) using meat from a commercial meat plants.

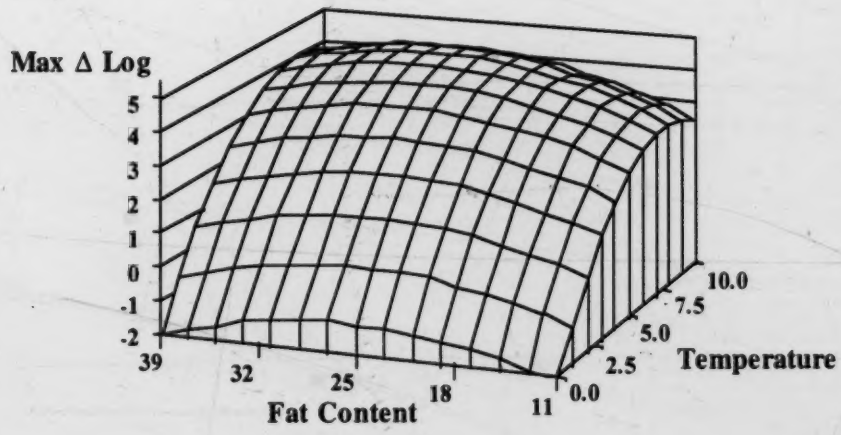


Figure 4.4. Effect of temperature of storage and fat content of the ground beef on maximum change in log CFU/g of *L. monocytogenes* (Max Δ log) during storage using meat from a commercial meat plant.

Fat content was significant at the 5% level when measuring Δ log CFU and was nonsignificant for max Δ log (Table 4.4).

The second order prediction equations (or estimating equations) for the response variables were generated using the regression coefficients from the SAS output of the data, and they are shown below:

$$\hat{Y} = -2.579 + 0.211X_1 + 0.159X_2 + 0.013X_1^2 + 0.003X_1X_2 + -0.003X_2^2 \quad (1)$$

$$\hat{Y} = -4.364 + 1.290X_1 + 0.255X_2 + -0.087X_1^2 + 0.004X_1X_2 + -0.005X_2^2 \quad (2)$$

Equation 1 is the prediction equation for Δ log CFU and equation 2 for the max Δ log. The relationship between the two independent variables and the dependent variable is shown in the three-dimensional response surface plots (Figures 4.3 and 4.4) that were generated from these prediction equations.

The response surface for Δ log CFU of *L. monocytogenes* of storage as a function of storage temperature and fat content of samples (shown in Figure 4.3) indicates that as temperature increased so did the response, maximizing at 10°C (maximum temperature studied). The response decreased as a function of temperature of storage at all levels of fat content of the samples. Similarly, an increase in fat content also resulted in an increase in the response; however, at fat content of about 30% or above the response remained unchanged. The effect of fat on growth of *L. monocytogenes* was not as great as the effect of temperature of storage. Temperature of storage was the most important factor because of contribution to sum of squares and fat content also influenced the response (Δ log CFU) but its contribution to sum of squares although significant was minor.

The response surface for max Δ log of *L. monocytogenes* as a function of temperature and fat content of samples (shown in Figure 4.4) indicates that regardless of fat content, the response increased with increase in temperature. Maximum response was seen at higher temperatures of storage. The effect of temperature was quadratic causing

curvature in the surface. There was no effect of fat content on the response. This was supported by the analysis of variance that showed fat content to be nonsignificant (Table 4.4).

The total counts obtained for all of the treatments were also analyzed to determine if the difference in growth of *L. monocytogenes* was due to differences in the growth of the background microflora. The background microflora of vacuum-packaged meats is mainly composed of lactic acid bacteria so total count can be presumed to be lactic acid bacteria. At 0 and 1.5°C there was no growth of the background microflora during 10 days of storage. At 5°C or above, the background microflora and *L. monocytogenes* grew with lag time of less than 3 days except for samples with 11% fat content that were stored at 5°C, in which case there was a lag time of 6 to 9 days (Figure 4.5). Max Δ log was also used as a response to determine the effect of the total count or lactic acid bacteria (Figure 4.6). In contrast to the *L. monocytogenes* response, in which max Δ log was markedly affected by temperature of storage (Figure 4.4), max Δ log for total count varied by approximately one log, indicating that the total count of the lactic microflora was minimally affected by the conditions of the experiment (Figure 4.6). *L. monocytogenes* counts and total count differed by about one log at temperatures of storage where *L. monocytogenes* grew, indicating that a major portion of total count was composed of *L. monocytogenes*; whereas, the difference was about 4 log in samples where *L. monocytogenes* did not grow. CCD was used to screen for important variables influencing growth of *L. monocytogenes* and results indicated that storage temperature was the most important variable.

RAPD of the colonies picked from PALCAM plates showed that all three strains of *L. monocytogenes* were detected in the final samples showing that all three strains grew in meat samples. A separate experiment was done in which ground beef samples (fat content 9%) inoculated with the same strains of *L. monocytogenes* were stored at 4°C. Results showed that maximum of 1.85 log increase in *L. monocytogenes* counts occurred which was in agreement with the predicted increase in number.

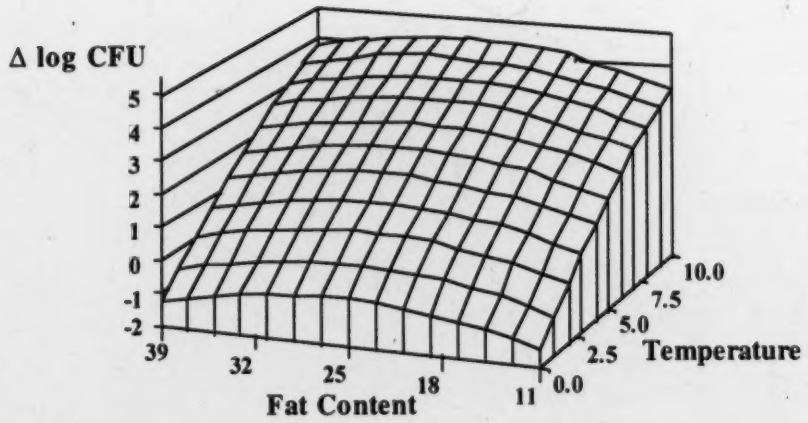


Figure 4.5. Effect of temperature of storage and fat content of the samples on the change of log CFU/g of total counts (Δ log CFU) in 10 days of storage using meat from a commercial meat plant.

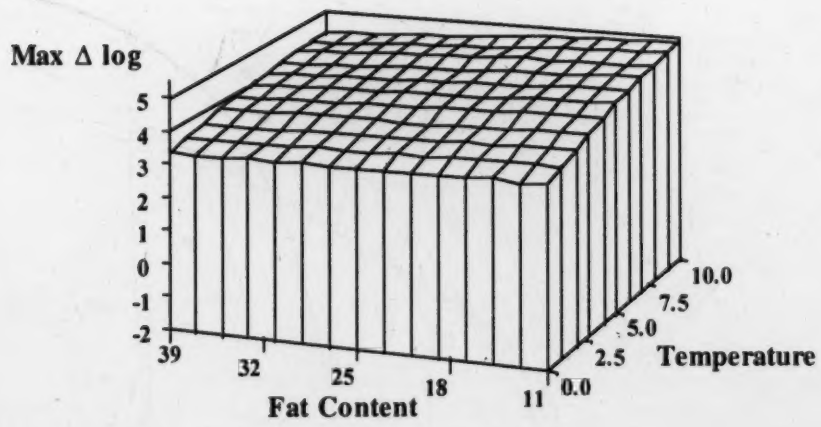


Figure 4.6. Effect of temperature of storage and fat content of the samples on maximum change in log CFU/g of total counts during storage (Max Δ log) using meat from a commercial meat plant.

4.4. DISCUSSION

In this study it was shown that growth of *L. monocytogenes* in vacuum-packaged ground beef was affected by storage temperature, fat content, and the natural microflora of the meat samples. Fat content and temperature of storage did not interact to affect the growth of these strains of *L. monocytogenes*. Temperature of storage was the main factor affecting growth of *L. monocytogenes* in the meat samples and fat content also affected growth of *L. monocytogenes*. For samples stored at 5°C (Figure 4.1B), the lower the fat content the longer the lag phase and the lower the maximum population of *L. monocytogenes*. Grau and Vanderlinde (1990) also reported that fatty tissue supported better growth of *L. monocytogenes* than lean meat. They suggested that the better growth on fatty tissue may be due to either pH being near neutrality or more available oxygen on the fat surface. Lean meat has more reducing ability than fat and this may have resulted in shorter lag phase of *L. monocytogenes* on fat. Growth of *L. monocytogenes* was observed at 4°C but not at 1.5°C, as a result the minimum temperature for growth of *L. monocytogenes* in our study was between 1.5 and 4°C. However, Grau and Vanderlinde (1990) reported growth of *L. monocytogenes* at 0°C. They grew the inocula at 10°C, inocula for this study were grown at 25°C and this might have resulted in very long lag phase of *L. monocytogenes*.

The difference in the results between the two experiments in this study showed that the natural (native) microflora can have a major effect on growth of *L. monocytogenes* in vacuum-packaged ground beef. Buchanan *et al* (1987) reported growth of *L. monocytogenes* in hamburger meat that was irradiated and suggested the possibility that the competitive microflora hindered growth of *L. monocytogenes*. This was also supported by Kaya and Schmidt (1991) who showed that on low pH beef the lactic acid bacteria became the dominant flora and suppressed the growth of *L. monocytogenes* while on high pH beef *Brochothrix thermosphacta* and *Enterobacteriaceae* dominated the microflora and *L. monocytogenes* was able to grow. Although bacteriocin-producing lactic acid bacteria were

isolated from the final samples in both of these studies, the lactics isolated from meat samples from the research abattoir were characterized as *Leuconostoc* sp. (*Leuconostoc* sp. UAL280) and formed a major component of the background microflora. In contrast, in the commercial meat samples bacteriocinogenic organisms were not the dominating population. In the present study the difference in the results between samples prepared from two sources of meat demonstrate that the composition and characteristics of the background microflora is a major factor determining the growth potential of *L. monocytogenes* in vacuum-packaged ground beef at refrigeration temperatures.

Ground beef products available in the market are likely to be contaminated with *L. monocytogenes* (Johnson *et al.*, 1990) and the results of the present study show that depending on the temperature of storage, fat content, and the competing microflora, growth of *L. monocytogenes* could occur at refrigeration temperatures. Although there are recommendations that the meat should be held at or below 0°C during longer storage times, there are studies that show the temperatures during retail display range from 1.7 to 10°C (Greer *et al.*, 1994). Depending on the competing microflora of the meat, there is the possibility that *L. monocytogenes* can grow to high numbers during extended storage. Growth of the pathogen to high numbers reaching up to 7 log CFU/g could lead to the risk of foodborne illness if the meats are not properly cooked or if the raw foods come in contact with the cooked products. This shows that growth of this foodborne pathogen should be controlled in uncooked meat products. Growth of *L. monocytogenes* can be controlled in vacuum-packaged ground beef with bacteria that are inhibitory to the pathogen and there are a number of bacteriocin-producing lactic acid bacteria that inhibit *L. monocytogenes*. This illustrates the potential for biopreservation with lactic acid bacteria that produce inhibitory agents against *L. monocytogenes*. This observation was also supported by the results of the study in which the background microflora dominated by lactic acid bacteria inhibited growth of *L. monocytogenes*. *L. monocytogenes* in vacuum-packaged ground beef is affected by temperature and fat content. In conclusion,

temperature is the main factor affecting the growth. Fat content also affects growth but its effect is not as marked as that of temperature. Background microflora also plays a major role in growth of *L. monocytogenes* in vacuum-packaged ground beef.

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5. Enhanced Safety of Vacuum-Packaged Ground Beef by *Leuconostoc gelidum* UAL187 and *Leuconostoc* sp. UAL280 Against *Listeria monocytogenes*

5.1. INTRODUCTION

Biopreservation is the enhancement of food safety and extension of storage life with the natural microflora (Stiles, 1996) and it has gained increasing attention as a means of "naturally" controlling growth of pathogenic and spoilage organisms in foods. Modified atmosphere packaged (MAP) meats with elevated levels of carbon dioxide or vacuum packaging increases the storage life of meats compared with aerobic storage (Egan and Shay, 1982; Borch and Nerbrink, 1989). Lactic acid bacteria (LAB) are the dominating bacteria on raw meats stored at refrigeration temperatures under such conditions (Egan, 1983; Dainty and Mackey, 1992). These organisms produce substances that are inhibitory to other microorganisms and thus, through competition and inhibition, LAB dominate anaerobically stored foods. *Lactobacillus*, *Leuconostoc* and *Carnobacterium* are the main genera of LAB that dominate anaerobically packaged meats (Shaw and Harding, 1984; Dainty and Mackey, 1992; McMullen and Stiles, 1993). To be successful as a biopreservative, the strain to be used should: compete with the background microflora of raw meats; not impart undesirable sensory changes; and inhibit pathogenic and spoilage microorganisms.

Bacteriocin-producing LAB are organisms that could be used for biopreservation. Bacteriocins are small peptides or proteins produced by bacteria which are generally active against closely related species (Klaenhammer, 1988). *Leuconostoc gelidum* UAL187 is a wild type bacteriocin-producing strain that was isolated from modified atmosphere packaged meats in our laboratory (Hastings and Stiles, 1991). It produces a plasmid-mediated and well-characterized bacteriocin, leucocin A (Hastings and Stiles, 1991; van Belkum and Stiles, 1995). Leisner *et al.* (1995) reported that *L. gelidum* UAL187-22, a

bacteriocin-producing partially cured variant of the wild type *L. gelidum* UAL187, could be used as a biopreservative culture to extend the storage life of beef slices. *L. gelidum* UAL187, a wild type isolate extends the storage life of meat by inhibiting a sulphide-producing strain of *Lactobacillus sake* (Leisner *et al.*, 1996). Worobo (1997) explored the potential of *L. gelidum* UAL187 as a biopreservative and reported that total LAB counts in samples inoculated with *L. gelidum* UAL187 were 1 log lower than in uninoculated samples and it improved the odour and red meat colour stability. These studies have shown that *L. gelidum* UAL187 can extend the storage life of raw meats but, further exploration of the potential of *L. gelidum* UAL187 to enhance the safety of raw meats is required.

Listeria monocytogenes is a foodborne pathogenic microorganism of concern because of its psychrotrophic nature and high mortality rate among high-risk populations such as unborn, newborn, or the immunocompromised (Marth, 1988). Meats with extended shelf-life may allow the growth of *L. monocytogenes* at refrigeration temperatures and it grows in ground beef at refrigeration temperatures including 5 and 10°C (Chapter 3). These temperatures are not uncommon in retail display cases. It is important therefore to find methods to control the growth of psychrotrophic pathogens in extended shelf-life meats. *L. gelidum* UAL187 produces a bacteriocin, leucocin A, that is active against a broad spectrum of other lactic acid bacteria, strains of *L. monocytogenes* and also against *Enterococcus* spp. *Leuconostoc* spp. UAL280 is a wild-type strain that was isolated from vacuum-packaged ground beef in which three strains of *L. monocytogenes* were unable to grow (chapter 3). The objective of this study was to investigate the biopreservative potential of the wild type, bacteriocinogenic (bac^+) and the nonbacteriocinogenic variant (bac^-) strain of *L. gelidum* UAL187 and wild type *Leuconostoc* sp. UAL280, against *L. monocytogenes* in vacuum-packaged ground beef at refrigeration temperatures.

5.2. MATERIALS AND METHODS

5.2.1. Bacterial Cultures and Media

LAB used in the study were *L. gelidum* UAL187 wild type bac$^+$ strain containing all three of its plasmids, pLG5.0, pLG7.6, and pLG9.2; *L. gelidum* UAL187-22, the bac$^+$ variant containing two plasmids pLG7.6 and pLG9.2; *L. gelidum* UAL187-13, the bac$^-$ variant, containing only plasmid pLG9.2 (Hastings and Stiles, 1991; van Belkum and Stiles, 1995); and *Leuconostoc* sp. UAL280 wild type strain that is bac$^+$ (Chapter 3). Stock cultures were frozen (-70°C) in APT broth (All Purpose Tween; Difco Laboratories Inc., Detroit, Mich.) containing 20% glycerol. The strains were maintained in Cooked Meat Medium (Difco) at 4°C and subcultured (0.1% inoculum) at least twice in APT at 25°C before use in experiments. A cocktail of three strains of *L. monocytogenes* was used in this study. Strains of *L. monocytogenes* and their growth conditions used in the study are described in detail in Chapter 3.

5.2.2. Ground Beef Preparation and Inoculation

Vacuum-packaged beef trim was obtained from a commercial meat plant (XL Meats, Calgary, Alberta) and stored at -25°C in a cooler until used in the experiment. Separate batches of trim were used for each replication. Before grinding, the meat was thawed at 4°C for 18 to 24 h. Meat was coarsely ground (10 mm plate) with a meat grinder (Omas TS12, Model TS8, Stoney Creek, Ontario). Fat content of the trim was determined by total lipid extraction (Folch *et al.*, 1956).

LAB inocula for all experiments were grown in 6 ml of APT broth (0.1% inoculum) and incubated at 25°C for 18 h before use in the experiments. *L. monocytogenes* strains were grown separately at 25°C in TSB-YE (Trypticase Soy Broth, BBL Microbiology Systems, Cockeysville, Maryland) containing 0.6% yeast extract, (Difco) and subcultured twice overnight at 25°C (0.1% inoculum) before use in the

experiment. One ml of an overnight culture of each strain was centrifuged at 16,000 × *g* for 4 min at 4°C, washed twice with 1 ml of sterile, 0.1% peptone water, resuspended and serially diluted with sterile, 0.1% peptone water to yield 10 ml of inoculum to produce an initial inoculum level of approximately 10^5 CFU per gram (CFU/g) for LAB and 10^3 CFU/g for *L. monocytogenes*. The inoculum was distributed over the surface of the ground meat on a sheet of aluminium foil. An equal amount of sterile, 0.1% peptone water was distributed over the coarse ground beef used as an uninoculated control. Grinder parts that would come in contact with meat were autoclaved before grinding. The meat was ground twice through a 6.7 mm plate.

5.2.3. Ground Beef Storage

Samples of 10 g of the ground beef were weighed and placed in bags made of high barrier film (Packall Packaging Inc., mylar/PVDC/polyethylene vinyl alcohol film; oxygen transmission rate 7.7 cc/m^2/24 h; moisture vapour transmission rate, 8.7 g/m^2/24 h). Each 10 g sample was vacuum-packaged (CVP Fresh Vac model A-300, CVP Systems Inc., Downers Grove, Illinois) and stored at either 5 or 10°C. Samples were stored for 28 days at 5°C and for 26 days at 10°C. Storage temperatures were monitored with Mirinz-Delphi temperature data loggers (Tru-Test Distributions Ltd., Auckland, New Zealand).

5.2.4. Bacteriology

At each sampling time two vacuum-packaged samples were blended separately with 90 ml of sterile, 0.1% peptone water for 2 min (Colworth Stomacher 400, Baxter Diagnostics Corp., Canlab Division, Edmonton, Alberta). Presumptive, indigenous LAB of the samples were enumerated on MRS agar after anaerobic incubation at 25°C for 72 h. *L. gelidum* UAL187, *L. gelidum* UAL187-13, and *Leuconostoc* sp. UAL280 were enumerated by spreading the appropriate dilutions of the serially diluted sample homogenates on the surface of prepoured plates of Lactobacilli MRS (MRS-S) agar

prepared according to the Difco formulation with dextrose replaced by 5% sucrose. Leuconostocs were enumerated by counting slime-producing colonies after anaerobic incubation at 25°C. Slime-producing colonies of control samples were counted to determine the slime-producing count of the indigenous microflora. Appropriate dilutions were spread on the surface of prepoured PALCAM Agar Base (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) supplemented with selective supplement (SR150E, Oxoid) to determine presumptive *L. monocytogenes* counts and on Plate Count Agar (PCA, Difco) to determine total aerobic plate counts. PALCAM plates were incubated aerobically at 37°C for 48 to 72 h and PCA plates were incubated at 25°C for 48 h. The mean log CFU for each sample was determined. pH of the 1:10 meat sample homogenates prepared for plate counting was measured at the time of sampling using a Fisher Accumet pH meter.

5.2.5. Antagonism of LAB against *L. monocytogenes* and Bacteriocin Detection in Meat Samples.

L. monocytogenes strains were checked for inhibition by bacteriocins produced by LAB by deferred inhibition test (Ahn and Stiles, 1990). Presumptive colonies of *L. monocytogenes* isolated from PALCAM plates at day 27 or 28 of storage were checked by deferred inhibition tests for resistance (Ahn and Stiles, 1990). A bacteriocin detection method described by Saucier (1997) was used to detect bacteriocin production in meat samples. Meat sample homogenates (1:10) prepared for plate counting were heated in a boiling water bath for 15 min and cooled on ice. These samples were centrifuged at 7970 × *g*, freeze dried, dissolved in 0.1% TFA and checked for bacteriocin by spot-on-lawn test against *L. monocytogenes* strains.

5.2.6. Experimental Design and Statistical Analysis

The experiment consisted of six treatments (1) an uninoculated control; and samples inoculated (2) inoculated with 3 log *L. monocytogenes*/g; (3) with 5 log *L. gelidum* UAL187-13; (bac^-) and 3 log *L. monocytogenes*/g; (4) 5 log *L. gelidum* UAL187 (bac^+)/g; (5) 5 log *L. gelidum* UAL187 (bac^+) and 3 log *L. monocytogenes*/g; (6) 5 log *Leuconostoc* sp. UAL280 and 3 log *L. monocytogenes*/g. The experiment was replicated with separate batches of meat. Replicate 1 had two extra treatments with inoculation of 5 log *L. gelidum* UAL187-13 (bac^-) and *Leuconostoc* sp. UAL280. Meat samples were stored at 5°C. Each treatment was tested in duplicate samples at each sampling time. Microbial counts and pH were subjected to analysis of variance according to General Linear Models procedure of the Statistical Analysis System (SAS, 1989). Student-Newman-Keuls' Multiple Range Test (Steel and Torrie, 1980) was used to determine the significance of the effect of drop of pH and inhibition of growth of *L. monocytogenes*.

To check the effect of LAB to control *L. monocytogenes* at an abusive storage temperature of 10°C, an experiment was done with treatments that were the same as in replication 1. Duplicate samples were used for each sampling time and the log mean of microbial counts was plotted against time of storage.

To observe the inhibitory action of LAB when they are inoculated at the same level as *L. monocytogenes* an experiment was designed with both LAB and *L. monocytogenes*, inoculated at 3 log CFU/g and stored at 5°C. *L. gelidum* UAL187-22, a bacteriocinogenic variant of *L. gelidum* UAL187 was used as a bacteriocin producing organism and not the wild-type organism. The treatments were prepared as (1) uninoculated control; (2) *L. monocytogenes*; (3) *L. monocytogenes* and *L. gelidum* UAL187-13; (4) *L. monocytogenes* and *L. gelidum* UAL187-22; (5) *L. monocytogenes* and 5 log CFU/g of *L. gelidum* UAL187-22; and (6) *L. monocytogenes* and *Leuconostoc* sp. UAL280.

5.3. RESULTS

Growth of the presumptive lactic microflora, as well as growth of the inoculated LAB and indigenous lactic microflora that produce slime from sucrose, is shown in Figure 5.1A and B. From the identical appearance of data for total microflora (data not shown) and presumptive lactic microflora (Figure 5.1A) it is apparent that the presumptive lactic acid bacteria were the dominant microflora. The presumptive lactic microflora was 2 log CFU/g in the control samples at time zero (Figure 5.1A) and they grew to 8 log CFU/g by day 12 of storage. The slime-producing indigenous lactic microflora in the meat grew to a maximum population of 6 to 7 log CFU/g by day 12 and was about 1 to 2 log CFU/g lower than total LAB counts (data not shown). The natural lactic microflora did not affect the counts of inoculated LAB because slime-producing LAB were the dominating bacteria in samples inoculated with LAB, reaching maximum population of 8 log CFU/g. There was a marked difference in the growth rates of *L. gelidum* UAL187-13 and *Leuconostoc* sp. UAL280 compared with that of *L. gelidum* UAL187. *L. gelidum* UAL187 reached 8 log CFU/g within 12 days of storage with standard deviations of ± 0.17 log CFU/g, while *L. gelidum* UAL187-13, and *Leuconostoc* sp. UAL280 grew faster and reached 8 log CFU/g by day 4 with standard deviations of ± 0.15 log CFU/g. Growth of LAB was similar in samples either inoculated alone or co-inoculated with *L. monocytogenes* (data not shown) and this indicated that *L. monocytogenes* had no effect on growth of LAB.

Growth and survival of *L. monocytogenes* inoculated at 3 log CFU/g is shown in Figure 5.2. *L. monocytogenes* was either not present or below 2 log CFU/g in samples that were not inoculated with *L. monocytogenes*. *L. monocytogenes* grew to 7 log CFU/g by day 12 of storage in samples with native microflora and remained at that level up to 28 days of storage with standard deviation less than ± 0.13 log CFU/g, indicating that the native microflora did not inhibit growth of *L. monocytogenes*. Growth of *L. monocytogenes* was significantly higher ($p < 0.05$) by a factor of 3 to 4 log cycles after 12 days of storage reaching counts of 7 log CFU/g in samples not co-inoculated with LAB

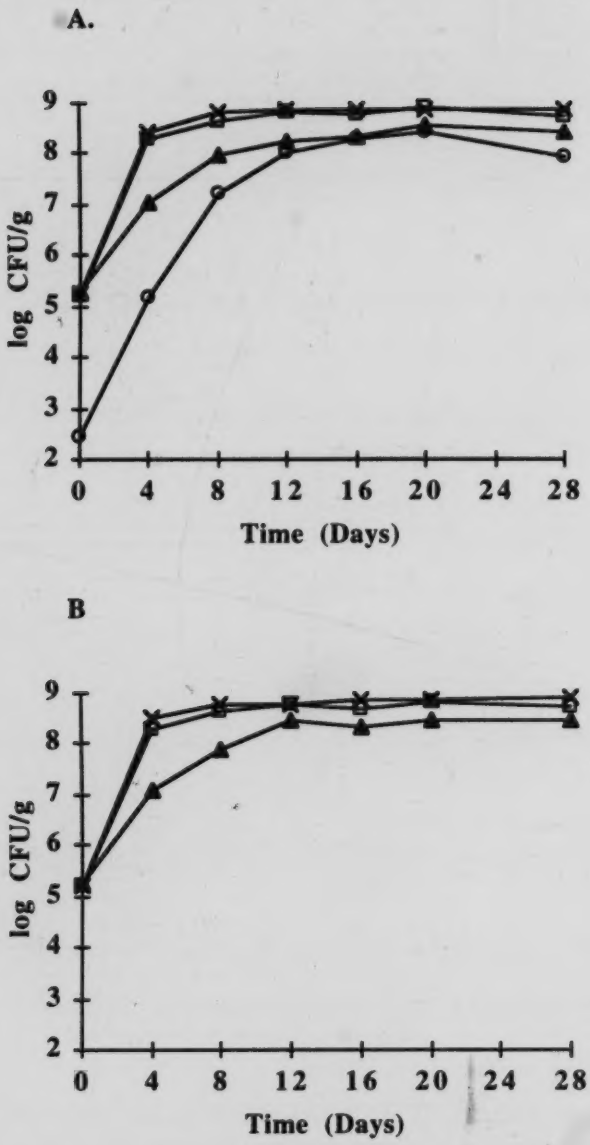

Figure 5.1. Growth of: (A) presumptive total lactic acid bacteria and (B) inoculated slime-producing lactic acid bacteria in vacuum-packaged ground beef using meat from a commercial meat plant and stored at 5°C with the following treatments: uninoculated control (O); and *L. gelidum* UAL187-13 (Δ); *L. gelidum* UAL187 (▲); and *Leuconostoc* sp. UAL280 (✖) when co-inoculated with *L. monocytogenes*. Leuconostocs were inoculated at 5 log CFU/g and *L. monocytogenes* at 3 log CFU/g. Each data point is the mean of four samples.

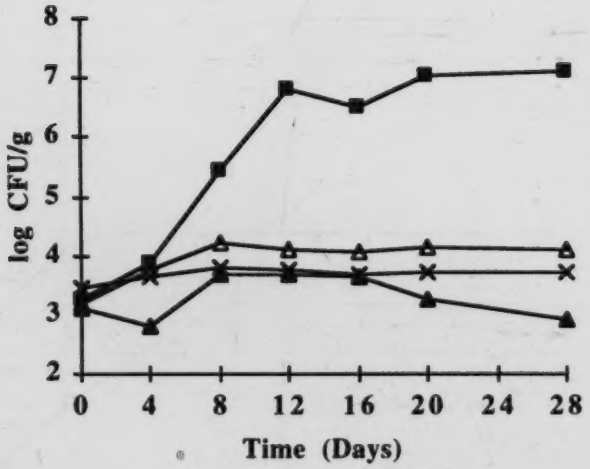

Figure 5.2. Growth and inhibition of *L. monocytogenes* in vacuum-packaged ground beef samples using meat from a commercial source and stored at 5°C with the following treatments: *L. monocytogenes* alone (■); and when *L. monocytogenes* co-inoculated with *L. gelidum* UAL187-13 (Δ); *L. gelidum* UAL187 (▲); and *Leuconostoc* sp. UAL280 (✖). Leuconostocs inoculation levels were 5 log CFU/g and *L. monocytogenes* at 3 log CFU/g. Each data point is the mean of four samples.

compared with counts of 3 to 4 log CFU/g in samples that were co-inoculated. There was a significant ($p < 0.05$) difference in *L. monocytogenes* populations between replications. This can be explained by differences in the lag times between two replications, it was more than 4 days of storage in replication 1 and less than 4 days in replication 2. There were significant differences ($p < 0.05$) in inhibition of *L. monocytogenes* by different LAB. By day 8 of storage, *L. monocytogenes* population had increased by 1 log CFU/g in the presence of *L. gelidum* UAL187-13, whereas in the presence of *L. gelidum* UAL187 and *Leuconostoc* sp. UAL280 the organism was still at the inoculation level of 3 log CFU/g.

There was a drop in pH of all samples including uninoculated samples (Figure 5.3 and there was no significant difference ($p > 0.05$) in the pH of all samples at day zero and by day 16 to 28 of storage, however, the relative time for drop in pH varied in different samples. By day 4 of storage, the pH was significantly lower ($p < 0.05$) in samples inoculated with *L. gelidum* UAL187-13 (bac^-) than in samples inoculated with *L. gelidum* UAL187 (bac^+). This coincided with the data for growth of the inoculated LAB, but *L. monocytogenes* counts remained the same in samples co-inoculated with *L. gelidum* UAL187-13 (bac^-), while in samples co-inoculated with *L. gelidum* UAL187 (bac^+) there was a significant decrease ($p < 0.05$) in *L. monocytogenes* counts. In spite of a significantly lower ($p < 0.05$) pH by day 12 there was 1 log growth of *L. monocytogenes* in the presence of bac^- variant of *L. gelidum* and no growth in the presence of the bac^+ strain. Bacteriocin was not detected in any of the samples after 4, 8, 12 or 20 days of storage. There was a significant difference ($p < 0.05$) of inhibition of *L. monocytogenes* by the bacteriocinogenic and nonbacteriocinogenic variants of *L. gelidum*. This suggests that there was an effect of bacteriocin on inhibition of *L. monocytogenes* and that there was significantly greater inhibition of *L. monocytogenes* even after 28 days of storage. *L. monocytogenes* colonies isolated at the end of replicate 1 were found to be resistant to bacteriocin produced by *L. gelidum* UAL187 but not in the case of replicate 2.

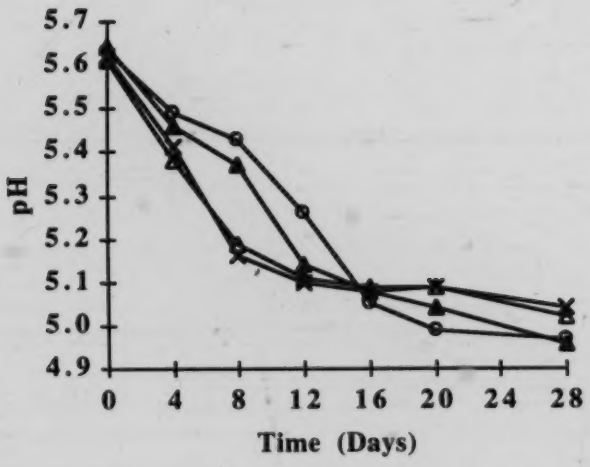

Figure 5.3. Change in pH of vacuum-packaged ground beef samples prepared with meat from a commercial source and stored at 5°C with the following treatments: uninoculated control (o); and in samples co-inoculated with *L. monocytogenes* and *L. gelidum* UAL187-13 (Δ); *L. gelidum* UAL187 (▲); and *Leuconostoc* sp. UAL280 (✖). Leuconostocs inoculation levels were 5 log CFU/g and *L. monocytogenes* at 3 log CFU/g. Each data point is the mean of four samples.

Even though resistant *L. monocytogenes* colonies were isolated in replicate 1, *L. monocytogenes* failed to grow in those samples.

Growth and survival of LAB and *L. monocytogenes* at the abusive storage temperature of 10°C is shown in Figures 5.4 and 5.5, respectively. The indigenous slime-producing lactics were lower than 8 log CFU/g after 26 days of storage, whereas, inoculated samples reached 8 log CFU/g by day 4 of storage. The indigenous lactic acid bacteria did not affect the counts of the inoculated LAB. Furthermore, the native microflora of the meat samples inoculated with *L. monocytogenes* only did not suppress the growth of *L. monocytogenes*. There was increase of 5 log CFU/g during 26 days of storage. Most of the growth occurred within 12 days of storage. The co-inoculation of meat samples with LAB lead to a marked suppression of *L. monocytogenes*. In the presence of *L. gelidum* UAL187 (bac^+) there was a 1 log decrease of *L. monocytogenes* counts; whereas, the *L. monocytogenes* counts remained at the inoculation level when they were co-inoculated with *L. gelidum* UAL187-13 (bac^-) (Figure 5.5). *Leuconostoc* sp. UAL280 also controlled the growth of *L. monocytogenes*, less than 1 log increase in *L. monocytogenes* occurred in the presence of UAL280 compared with 5 to 6 log increase of *L. monocytogenes* in control samples.

Growth of LAB and *L. monocytogenes* inoculated at the same level is shown in Figures 5.6 and 5.7, respectively. The background microflora at day zero was higher (4 log CFU/g) than inoculation level of LAB (3 log CFU/g) (data not shown). *L. gelidum* UAL187-13 and *Leuconostoc* sp. UAL280 grew faster and reached maximum population level of 8 log CFU/g within 8 days of storage (Figure 5.6) and they dominated the microflora of meat samples, because in control samples the lactic microflora only reached 8 log CFU/g within 12 days of storage (data not shown). *L. gelidum* UAL187-22, a bacteriocin-producing variant with a slower growth rate than wild-type *L. gelidum* UAL187 (Leisner *et al.*, 1995), reached counts of 8 log CFU/g sometime between 16 and 26 days of storage when inoculated at an initial level of 3 log CFU/g.

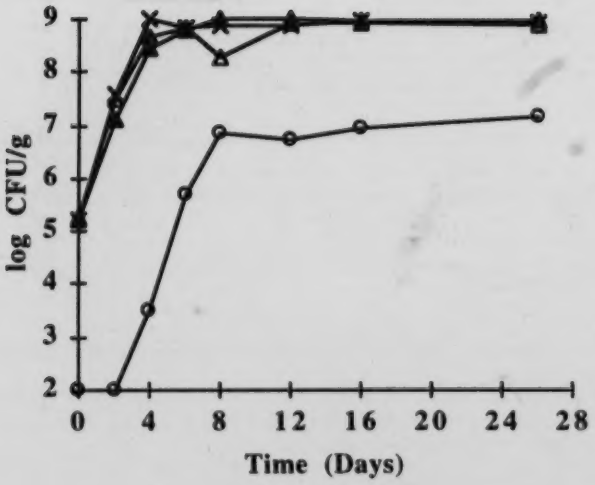

Figure 5.4. Growth of inoculated and indigenous slime-producing LAB in vacuum-packaged ground beef samples stored at 10°C and prepared with meat from a commercial meat plant with the following treatments: uninoculated control (o); and *L. gelidum* UAL187-13 (Δ); *L. gelidum* UAL187 (▲); and *Leuconostoc* sp. UAL280(✖) when co-inoculated with *L. monocytogenes* . Leuconostocs inoculation levels were 5 log CFU/g and *L. monocytogenes* at 3 log CFU/g. Each data point is the mean of two samples.

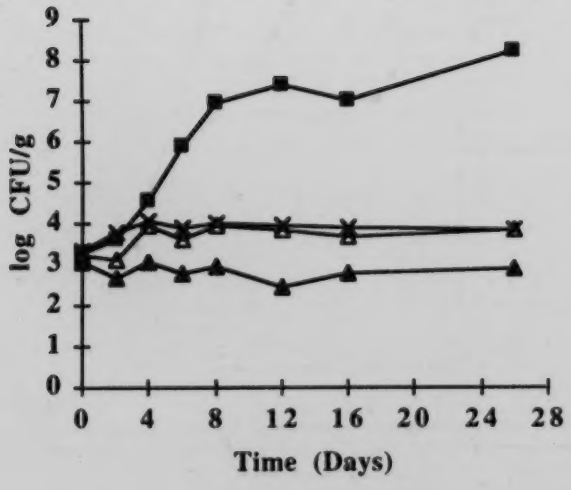

Figure 5.5. Growth and survival of *L. monocytogenes* in vacuum-packaged ground beef samples stored at 10°C and prepared with meat from a commercial plant with the following treatments: *L. monocytogenes* alone (■); and when co-inoculated with *L. gelidum* UAL187-13 (Δ); *L. gelidum* UAL187 (▲); and *Leuconostoc* sp. UAL280 (✖). Leuconostocs inoculation levels were 5 log CFU/g and *L. monocytogenes* at 3 log CFU/g. Each data point is the mean of two samples.

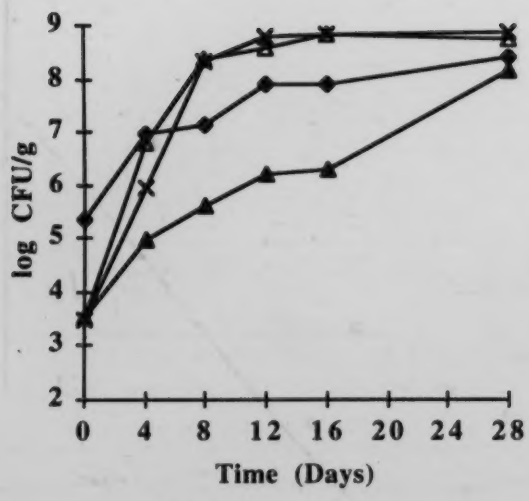

Figure 5.6. Growth of inoculated slime-producing LAB in vacuum-packaged ground beef samples stored at 5°C and prepared with meat from a commercial meat plant with the following treatments: *L. gelidum* UAL187-13 at inoculation level of 3 log CFU/g (Δ); *L. gelidum* UAL187-22 at inoculation level of 3 log CFU/g (▲); *L. gelidum* UAL187-22 at inoculation level of 5 log CFU/g (◆) and *Leuconostoc* sp. UAL280 (✖) at inoculation level of 3 log CFU/g when co-inoculated with *L. monocytogenes* inoculated at 3 log CFU/g. Each data point is the mean of two samples.

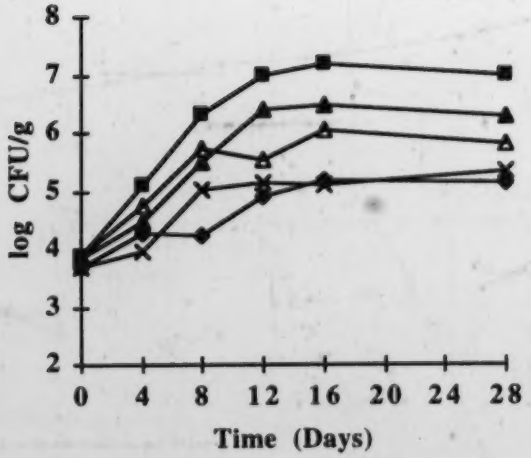

Figure 5.7. Growth and survival of *L. monocytogenes* in vacuum-packaged ground beef samples stored at 5°C and prepared with meat from a commercial plant in treatments: *L. monocytogenes* alone (■); and *L. monocytogenes* when co-inoculated with *L. gelidum* UAL187-13 at inoculation level of 3 log CFU/g (Δ); *L. gelidum* UAL187-22 at inoculation level of 3 log CFU/g (▲); *L. gelidum* UAL187-22 at inoculation level of 5 log CFU/g (◆) and *Leuconostoc* sp. UAL280 (✖) at inoculation level of 3 log CFU/g. *L. monocytogenes* was inoculated at 3 log CFU/g. Each data point is the mean of two samples.

The background lactic microflora grew faster than this LAB (data not shown). When *L. gelidum* UAL187-22 was inoculated at 5 log CFU/g it grew slower than UAL187-13 inoculated at 3 log CFU/g and reached maximum population of 8 log CFU/g by day 12 of storage, while *L. gelidum* UAL187-13 inoculated at 3 log CFU/g reached the same level by day 8.

Growth of *L. monocytogenes* was not inhibited by background microflora and there was 3 to 4 log increase in counts of *L. monocytogenes* reaching 7 log CFU/g by day 16 of storage. *L. monocytogenes* grew in the presence of all LAB but not to the same population levels as that of the control samples. The level of inhibition of *L. monocytogenes* was different by different LAB. Both *Leuconostoc* sp. UAL280 inoculated at 3 log CFU/g and *L. gelidum* UAL187-22 inoculated at 5 log CFU/g caused inhibition of *L. monocytogenes* to the same levels and it was 2 log lower than in the control. Inhibition of *L. monocytogenes* was about 0.5 log less by *L. gelidum* UAL187-22 than by *L. gelidum* UAL187-13 inoculated at the same levels of 3 log CFU/g where *L. monocytogenes* grew about 2 to 3 log.

5.4. DISCUSSION

L. gelidum UAL187 (bac^+), *L. gelidum* UAL187-13 (bac^-), and *Leuconostoc* sp. UAL280 inhibited growth of *L. monocytogenes* in vacuum-packaged ground beef at 5 and 10°C, whereas the indigenous microflora of the meat did not inhibit growth of *L. monocytogenes*. In spite of relatively faster growth of *L. gelidum* UAL187-13 (bac^-), populations of *L. monocytogenes* were lowered to a greater extent with *L. gelidum* UAL187 (bac^+). At the end of the study, after 26 days of storage, there was about 1 log difference in *L. monocytogenes* populations depending on the strain of *Leuconostoc* inoculated onto the meat at 5 log CFU/g. The drop in pH was greater with *L. gelidum* UAL187-13 (bac^-) and inhibition was greater by *L. gelidum* UAL187 (bac^+). So it could be concluded that bacteriocin-producing LAB were more effective in controlling growth of

L. monocytogenes than the isogenic nonbacteriocin-producing variant. Leuconostocs utilize glucose rapidly when stored anaerobically (Borch and Agerhem, 1992) and because the leuconostocs were inoculated at 2 log CFU/g higher than *L. monocytogenes*, they might a have competitive advantage for available glucose. So the suppression of *L. monocytogenes* by *L. gelidum* UAL187-13 (bac^-) probably may be due to competition for available energy source and effect of the pH. However, when the bacteriocinogenic strain *L. gelidum* UAL187 (bac^+) was used there was an additional drop in listeriae counts that may have been due to bacteriocin production. *L. gelidum* UAL187 is an early producer of bacteriocin (Ahn and Stiles, 1990). Use of the nonbacteriocinogenic isogenic variant (UAL187-13) confirmed the role of bacteriocin in control of *L. monocytogenes*. Inhibition of the pathogen cannot be explained by a drop in pH because inhibition was observed when pH was above 5.0 and growth of *L. monocytogenes* at pH values less than 5.0 has been reported (George *et al.*, 1988). A nonbacteriocinogenic variant of *Leuconostoc* sp. UAL280 was not available so it is difficult to interpret whether the inhibition of *L. monocytogenes* was due to bacteriocin or competition for nutrients, but the results of this study showed that this strain was also effective for controlling growth of *L. monocytogenes* in vacuum-packaged ground beef. Inhibition of *L. monocytogenes* was also observed when meat was stored at 10°C, showing that the inoculated LAB controlled growth of *L. monocytogenes* at abusive retail temperatures. The level of inhibition of *L. monocytogenes* by LAB inoculated at 3 log CFU/g was not as marked as when LAB inoculated at 2 log higher than *L. monocytogenes*. Even *L. gelidum* UAL187-22, a slow growing variant (Leisner *et al.*, 1995), caused inhibition of *L. monocytogenes*.

L. monocytogenes can be found in retail meats up to 3 log CFU/g (Johnson *et al.*, 1990) and inoculation of 3 log CFU of *L. monocytogenes*/g was used in the present study. Use of a cocktail of three strains of *L. monocytogenes* reduced the chances of basing the conclusions about inhibition of *L. monocytogenes* on strain sensitivity as different strains may have different sensitivity to the bacteriocins produced by LAB. Emergence of resistant

strains is one of the concerns about the use of bacteriocinogenic LAB. There were resistant strains of *L. monocytogenes* isolated from meat samples inoculated with bacteriocinogenic *L. gelidum* in replicate 1 and not from replicate 2. In spite of the presence of the resistant strains listeriae failed to grow in the presence of inoculated LAB. It could not be concluded whether resistance had any effect on growth of *L. monocytogenes* or not. Even the nonbacteriocinogenic variant was sufficient to inhibit *L. monocytogenes* growth in the meat. The fact that inoculated LAB should be competitive and fast growing is also supported by less inhibition by *L. gelidum* UAL187-22 which grew slowly in meats. This study supports the use of *L. gelidum* to ensure the safety of such meats because the indigenous microflora failed to inhibit growth of *L. monocytogenes*. The results of this study have shown that *L. gelidum* UAL187 and *Leuconostoc* UAL280 have the capacity to provide the additional "hurdle" for growth of *L. monocytogenes*. Most bacteria are killed by normal cooking, but, for raw meats, if growth of the pathogenic organism to high levels is inhibited, the risk of survival through undercooking or cross-contamination of cooked products is reduced.

Leuconostocs are generally not considered suitable for meats because of slime production in the presence of sucrose, but *L. gelidum* UAL187 is a good candidate for biopreservation of vacuum-packaged ground beef because it controls spoilage by *L. sake* (Leisner *et al.*, 1996), extends the storage life by stabilizing odour and red colour (Worobo, 1997), and it has now been shown that it enhances the safety at refrigeration temperatures. *Leuconostoc* sp. UAL280 also enhances the safety of such meats but this organism must still be tested for its effect on storage life of refrigerated meats.

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6. General Discussion and Conclusions

Listeria monocytogenes has become an important public health problem and a bacterium of great concern to the food industry because of its recognition as a foodborne pathogen with serious consequences for susceptible individuals. The different characteristics that make *L. monocytogenes* an organism of importance for food industry are its psychrotrophic nature, high mortality rate associated with documented cases of foodborne illness and increasing evidence that sources of contamination of foods is likely to originate from the environment (Jones, 1990; Farber and Peterkin, 1991). *L. monocytogenes* has been found in a variety of foods including raw meats, vegetables, processed meat, and dairy products. Johnson *et al.* (1990) reviewed the incidence of *L. monocytogenes* in meat and meat products and reported that it is a common contaminant of such products and that in ground beef the listeriae can range from 8 to 92%. In Canada 58% of the samples contained *L. monocytogenes*. The presence of *L. monocytogenes* in processed foods is primarily due to cross-contamination and not by its heat resistance, because *L. monocytogenes* is killed by pasteurization and normal cooking temperatures that are used to prepare ready-to-eat processed meats.

The ubiquitous nature of the organism makes it difficult if not impossible to keep foods free of these pathogenic bacteria and it was concluded by experts in the World Health Organization's informal working group on listeriosis (WHO, Working Group, 1988) that "total elimination of *L. monocytogenes* from all food is impractical and may be impossible." Different policies on the presence of *L. monocytogenes* in foods have been adopted by different countries. The United States Food and Drug Administration (FDA), the U. S. Department of Agriculture (USDA), and Centers for Disease Control and Prevention (CDC) have agreed that there should be a "zero tolerance" for *L. monocytogenes* in foods not intended for further heat treatment (McNamara, 1994), but Canadian policy depends on good manufacturing practices during processing of foods.

Listeriosis, the infection caused by *L. monocytogenes*, can be prevented by thorough cooking of raw foods of animal origin and by separating raw and cooked foods.

Aerobic storage life of red meats is a few days because of growth of the aerobic spoilage microflora that causes spoilage before psychrotrophic pathogenic bacteria can grow. In contrast, anaerobic and vacuum-packaging is used to extend storage life of ground beef because carbon dioxide has a bacteriostatic effect on some bacteria (Dixon and Kell, 1989; Zhao *et al.*, 1994) and nonputrefactive lactic acid bacteria dominate the microflora. Concerns have been expressed about microbiological risks posed by extended, refrigerated storage of foods in anaerobic or vacuum packaging (Farber, 1991; Lambert *et al.*, 1991) because psychrotrophic, facultatively anaerobic pathogenic foodborne bacteria, such as *L. monocytogenes* can grow to high levels before spoilage occurs.

LAB are the organisms that dominate the microflora of anaerobically packaged, chill stored ground beef because of their ability to survive the bacteriostatic effects of carbon dioxide and to grow at refrigeration temperatures. It is well known that LAB produce inhibitory substances when they grow in foods. When aerobic putrefactive spoilage microflora are replaced by LAB, storage life is extended because spoilage by LAB generally occurs after maximum population of LAB is reached. A predictable storage life of fresh meat could be obtained by inoculating the meats with known LAB that are inhibitory to other unwanted microorganisms. A wide range of bacteriocins produced by lactic acid bacteria is known to inhibit other lactic acid bacteria and pathogenic organisms such as *L. monocytogenes* (Harris *et al.*, 1989). LAB can be used for natural preservation because they provide an additional "hurdle" for the spoilage and pathogenic organisms through competition and inhibition. As the number of barriers is increased it becomes more difficult for the undesirable target organisms to grow or survive (Leistner, 1994). *Leuconostoc gelidum* UAL187 is a bacteriocinogenic LAB that was isolated from chill-stored meats and it has been proposed as a potential biopreservative organism (Leisner *et al.*, 1995). Subsequently *L. gelidum* UAL187 was shown to extend storage life of raw

ground beef at refrigeration temperatures when co-inoculated with sulphide-producing *Lactobacillus sake* (Leisner *et al.*, 1996) and to improve odour and to stabilize colour of ground beef (Worobo, 1997). Studies on the control of *L. monocytogenes* in such meats have not been done. *L. monocytogenes* is associated with raw meats and as a result the overall objective of the present study was to investigate growth of *L. monocytogenes* in vacuum-packaged ground beef, and if growth occurs, to control it with bacteriocinogenic LAB.

Reports on the growth of *L. monocytogenes* in raw meats stored at refrigeration temperatures have given rise to conflicting results. Growth depends on strain of *L. monocytogenes* used, pH of the meat (Barbosa *et al.*, 1995), type of tissue (fatty or lean), storage temperature (Grau and Vanderlinde, 1990), and background microflora (Kaya and Schmidt, 1991). In this study, the effect of temperature over a range of refrigeration temperatures (retail temperatures of 10°C are not uncommon) and fat content of ground beef of normal pH of 5.5 to 5.7 was studied. A cocktail of three strains of *L. monocytogenes*, that were originally isolated from meat, or meat processing equipment were used to inoculate meat. The cocktail of three strains was used to reduce the effect of strain differences and the strains were shown to be compatible, i.e., they were not inhibitory to each other. To trace the strains inoculated in the cocktail, random amplification of polymorphic DNA (RAPD) was used as a method of strain differentiation. RAPD allowed differentiation of the strains in this study which were indistinguishable by serotyping and plasmid profiling techniques.

Two factorial central composite design was used to investigate the effect of storage temperature and fat content of samples on the growth of *L. monocytogenes* in chill stored vacuum-packaged ground beef. Interesting results about growth of *L. monocytogenes* were obtained in meat samples prepared from meat from a research abattoir. Growth of *L. monocytogenes* was inhibited and only 1 log CFU/g increase in *L. monocytogenes* counts was observed even at a storage temperature of 10°C. This lead to the isolation of

bacteriocinogenic lactic acid bacteria from the samples and the results showed that the indigenous microflora was dominated by LAB that were inhibitory to all three of the inoculated strains of *L. monocytogenes*. It was speculated that the reason for inhibition of growth of *L. monocytogenes* was the growth of these LAB. Another experiment with same treatment combinations was done with meat from a commercial abattoir. Interestingly, *L. monocytogenes* grew in these samples, reaching numbers of 7 log CFU/g from an initial inoculation level of 3 log CFU/g. Bacteriocin-producing organisms were also isolated from samples of this study, but they were not the dominating microflora. There are studies that have reported the effect of background microflora on growth of *L. monocytogenes*, but they used meat of low (<6.0) and high (>6.0) pH levels and concluded that low pH of meats selected for growth of LAB and resulted in inhibition of *L. monocytogenes* (Kaya and Schmidt, 1991). But the present study showed that even in normal pH meat, growth of *L. monocytogenes* can occur and that growth depends on the type or composition of indigenous microflora. Storage temperature and fat content of the samples affected growth of *L. monocytogenes* but no interaction between the two factors was found. Storage temperature was the major factor affecting growth of *L. monocytogenes* in ground beef. Growth occurred at higher storage temperatures and fat content affected growth at those temperatures. RAPD results showed that all three strains grew in samples or survived where no increase of *L. monocytogenes* occurred during the storage time.

Results of this study showed that storage of vacuum-packaged ground beef at refrigeration temperatures may not prevent growth of *L. monocytogenes* during extended storage. The objective of the second study was to investigate if the growth of *L. monocytogenes* could be controlled in chill stored, vacuum-packaged ground beef by *L. gelidum* UAL187 (bacteriocinogenic), its isogenic variant *L. gelidum* UAL187-13 (nonbacteriocinogenic), and *Leuconostoc* sp. UAL280 (bacteriocinogenic). All three of these LAB controlled the growth of *L. monocytogenes* in vacuum-packaged ground beef at refrigeration temperature of 5°C and an abusive retail temperature of 10°C compared with

the uninoculated control samples. Growth of the pathogen was inhibited by *L. gelidum* UAL187-13 allowing a maximum increase *L. monocytogenes* of only 1 log CFU/g. Inhibition of growth by *L. gelidum* UAL187 was significantly greater ($p < 0.05$) allowing no increase in counts of *L. monocytogenes* during 28 days of storage. This greater level of inhibition was attributed to bacteriocin production (unfortunately bacteriocin was not detected in meat samples). *Leuconostoc* sp. UAL280 also prevented growth of *L. monocytogenes* and thus supported the theory that inhibition of growth in first study using meat from a research abattoir was caused by this LAB. Because of the absence of a nonbacteriocinogenic variant of this organism, it was not possible to attribute inhibition to bacteriocin activity. Because of concerns about the emergence of resistant strains of *L. monocytogenes*, the *L. monocytogenes* surviving at the end of storage period were tested for resistance. In replication 1, resistant strains to the bacteriocin produced by *L. gelidum* UAL187 were detected but not from replication 2. The colonies isolated from samples co-inoculated with *L. gelidum* UAL187-13 and *Leuconostoc* sp. UAL280 did not result in the appearance of resistant strains. The development of resistance is one of the concerns about the use of bacteriocin-producing LAB and it needs further clarification.

L. gelidum UAL187 is an important LAB with biopreservative properties for vacuum-packaged ground beef stored at refrigeration temperatures. This organism meets all of the prerequisites for biopreservation, i.e., it extends storage life by delaying spoilage, it does not result in undesirable sensory attributes (Worobo, 1997), and it ensures safety against psychrotrophic pathogenic bacteria such as *L. monocytogenes*. Ground beef is not consumed raw and *L. monocytogenes* should be killed by normal cooking practices. But listeriosis has been linked to consumption of uncooked hot dogs or undercooked poultry (Schwartz *et al.*, 1988). Because we have shown that vacuum-packaged ground beef can support growth of *L. monocytogenes* to large numbers at refrigeration temperatures, *L. monocytogenes* could pose a health risk due to survival in, or cross-contamination of, cooked meat. If the vacuum-packaged ground beef is inoculated with these LAB, growth

of *L. monocytogenes* will be prevented and therefore, chances of foodborne listeriosis resulting from consumption of undercooked red meats or from cross-contamination of cooked foods will be reduced.

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